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Electron Microscopic Studies on the Epidermis of Newt with an Enquiry into the Problem of Induced Neoplasia

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Electron Microscopic Studies on the Epidermis of Newt with an Enquiry into the Problem of Induced Neoplasia

By

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With 59 Figures

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I. Introduction

In what we call normal state, the constituent cells of a tissue maintain a dynamic equilibrium amongst themselves as well as with contiguous cells or the rest of the body. This equilibrium may be disturbed during reactions to abnormal stimuli; it may, however, be re-established when conditions are restored to normal. In other words, the cells in question have not undergone any fundamental or permanent change in the course of their reaction to and recovery from these abnormal stimuli. In some other circumstances, the equilibrium may be irreversibly affected. In such cases, certain permanent changes could be provoked in the cells which undermine the latter's capacity to fulfill their normal functions as integrated units in the body. This is especially the case when the stimuli happen to be carcinogens: the outcome is cancer.

How cellular equilibrium is maintained in normal state is as little understood as how it becomes upset in cancer. However, the importance of surface conditions in relation to intercellular affinities is being recognised more and more (Berrill 1945; Coman 1944 and 1955; Tyler 1947; P. Weiss 1947 and 1958; Holtfreter 1948; Spiegel 1954; Cowdry 1955; Devillers 1955; Abercrombie, Heaysman, and Karthausen 1957; Abercrombie and Ambrose 1958; Huxley 1958; L. Weiss 1960). As the cell surface is unavoidably involved in all intercellular relations, whatever may be the other influences that condition normality, it is to this organelle, as P. Weiss (1947) points out, that "we must look for the revelation of the factors that make or break specific cellular associations."

Our knowledge of intercellular associations is much clarified by the advance of electron microscopy. As its techniques become more refined, the time seems appropriate now to attempt comparative evaluations of the differences in these associations that may underlie different behavioural patterns of cells and tissues. This is the idea that prompted the present enquiry. Epidermal tissue with its peculiar intercellular relationships seemed to offer a suitable material. Contiguous cells make specific appositions through specialised regions of the plasma membrane. These specialised areas of specific contacts are agreed by all workers to serve firm attachment of cells necessary for the proper integration of the tissue. These have further been presumed to show deviations from the normal in the cancerous epithelium on theoretical grounds (Pillai 1957).

The crested newt *Triturus cristatus* Laurenti was selected as the animal to provide materials for the investigation. The epidermis of this animal was first shown by Koch, B. Schreiber and G. Schreiber (1939) to react to tar and benzopyrene producing infiltrating hyperplasia. This fact was confirmed and clarified by the more elaborate study of Neukomm (1944). He has shown that the epidermis of the newt reacts to subcutaneous injection of carcinogens in oil solution by producing neoplasia in about six days. This infiltrates into deeper tissues breaking through the basement membrane. 'Around three months' time the tissue regains normal organisation. This reaction of the epidermis of the newt thus presents two important points of interest to a student of carcinogenesis: it simulates carcino-

genesis by developing pronounced neoplasia which infiltrates and invades the deeper tissues; this neoplasia is temporary and regresses completely—a behaviour, anyone would wish to see the cancers assume! The facility with which this reaction can be produced and its fine sensibility are being exploited for testing and evaluating the carcinogenicity of substances (Neukomm 1957 and 1959). Various studies from diverse angles are now carried on in several laboratories to elucidate this curious problem of epidermal neoplasia in the newt. Histologically, the cellular components of this neoplasia are reported to have a normal appearance (Neukomm 1944). The apparent incongruity between this histological picture of normal structure and biological behaviour of active proliferation and invasion was thought an interesting problem to resolve by means of electron microscopy. The present study being one of the first attempts, is concerned with assessing the utility of such an ultrastructural analysis as it may shed some useful light on the problems of malignant growth. So instead of restricting the subject to a more complete treatment of any one aspect (e.g., fine structure of the normal epidermis of the newt), a rapid survey of the tissue in normal as well as experimentally altered conditions was undertaken with an enquiry into the structural modifications entailed during the early stages of the development of neoplasia.

Though the amphibian tegument has been the material for many recent electron microscopic investigations (Ottoson, Sjöstrand, Stenström and Svaetichin 1953; Porter 1956; Weiss and Ferris 1954a, b, and 1956; P. Weiss 1956; Millonig 1958; Salpeter and Singer 1959 and 1960; Kemp 1959; Hay 1960 and Schmidt 1960), a general description of the fine structure of the epidermis has not been attempted. Therefore, the first task here is to describe in some detail the ultrastructure of the normal tissue. This is to include also brief characterisation of the various kinds of atypical cells described by classical cytologists in the amphibian epidermis (Rabl 1951). Special attention is paid to intercellular relationships; the results of some simple treatments carried out with the intention of trying to alter them are given in this connection. Finally, the above mentioned observations are compared to those on neoplasia induced by subcutaneous injections of methylcholanthrene in ground-nut oil. Special controls are provided by observations on tissues taken from animals injected with only pure ground-nut oil.

Some preliminary results of this work have already been published (Pillai, Guénin and Gautier 1960; Pillai and Gautier 1960). In order to limit the number of figures reprinted here, references to the illustrations in these publications are often made.

I wish to express my thanks to Prof. H.-A. Guénin, of the Zoological Laboratory, to Mr. A. Gautier, Head of the "Centre de Microscopie Electronique" and to Dr. S. Neukomm, Director of the "Service des recherches expérimentales du Centre Anticancéreux Romand" for their guidance, to the Scientific Advisory Committee of the Lady Tata Memorial Trust in London for the award of an international scholarship and to the "Fonds National Suisse de la Recherche Scientifique" for a grant supporting a research program with which this work was connected.

Last, but not least, I am most happy to record here my great indebtedness to Prof. R. Choksi of the Tata Trusts, Bombay, who found me the first opportunity to enter into Scientific Research and who has been a source of encouragement and help ever since.

II. Materials and Methods

Newts of the species *Triturus cristatus* Laur. were obtained from Italy. Epidermal tissue was excised from the proximal half of the tail of the animals for all observations. Since this tissue shows no apparent differences in the male and the female, both sexes were used indifferently.

The fixed and processed tissue used for ultramicrotomy and electron microscopy is here referred to as a "preparation." Each preparation yielded about 5 to 15 tissue-blocks. The majority of preparations were made from different animals. Thus, 45 animals provided material for 57 preparations which comprise: control tissues from non-treated animals (16); tissues treated (*in vivo* and *in vitro*) with heterotonic solutions and chelating agent (22); tissues from oil-injected animals (4); and tissues from carcinogen-injected animals (15). In addition, 21 fixations were made for exclusive optical studies.

Many preparations of normal epidermis were made from tissues removed by simple hand-scraping with a razor blade or scalpel. Complete anaesthesia was given sometimes by keeping the animals for about 10 minutes in 0.1% solution of Tricaine (a product of Sandoz A. G., Basle, Switzerland) to facilitate handling. This treatment is inconsequential as regards notable structural alterations. Concerning neoplasia, though it is easily observable in stained optical preparations, its exact localisation in the living animal is extremely difficult. The method employed for its excision is as follows. The point of injection of the carcinogen was indicated by passing a thread through the ventral fringe of the tail of the animal. Excision was performed at a particular distance away from the thread, which place was supposed to include neoplasia or its immediate portions. After trying hand-scraping of the skin from anaesthetised animals for the first two preparations, a high-speed rotary punch (with needles of internal diameter 0.9 or 1.3 mm) was employed following the indication of Clark and Hibbs (1958). The punch was allowed to pass through the whole thickness of the tail, on either side of which neoplasia was supposed to have developed following injections of the carcinogen. Epidermal tissue from either side of the punched piece was cut off rapidly with a new razor blade while immersed in the fixing medium. During the whole operation of punching, physiological saline was poured over the area; the punched piece was also first ejected into the saline by a syringe which was filled with the same fluid. The saline solution was employed to avoid the sticking of the punch-tissue inside the needle and facilitate its ejection by working the syringe. The portion of the tail bearing the punch-hole was cut off, fixed and processed for serial sectioning to ascertain optically whether the punch really passed through the neoplasia.

The problem of the orientation of tissues was first solved in the following manner: pieces of excised skin of about 2 to 3 mm in diameter were carefully stretched over paraffin in short glass tubes, with the help of fine entomological pins, the whole operation being performed under the dissecting microscope. The pieces thus stretched were flooded with the fixative. After the fixation was complete, they were easily cut into smaller pieces whose orientation was checked during embedding. But later it was found easier and more profitable to allow very small pieces without any previous stretching, to take random orientation

inside the polyester-filled gelatin capsules. Before proceeding for ultramicrotomy, fine hand-made shavings of the four sides of the tissue-block together with semi-fine sections by the microtome were taken for optical control studies (Gautier 1960). This enabled slight changes of orientation when the blocks were chipped for ultramicrotomy. Exact knowledge of orientation was obtained from stained preparations of hand-made as well as microtome-sections.

Excepting three, all the other tissue-specimens were fixed in either 1% or 2% solution of osmium tetroxide buffered to a pH of 7.3–7.4 (Palade 1952). The fixations were carried out at room temperature, the duration being dependent on the strength of the solution: two hours for 1% and one hour for 2%. The three exceptions were those fixed in 0.6% ice-cold potassium permanganate solution buffered to the same pH value (Luft 1956); the fixation continued for two hours in an ice-bath. For exclusively optic studies, either Bouin's fluid or Duboscq-Brasil's was used.

Excepting the tissues embedded in methacrylate, which were dehydrated in graded series of alcohol, the rest which were embedded in polyesters were dehydrated in graded series of acetone according to Kellenberger, Schwab and Ryter (1956). Out of the 57 preparations, 49 were embedded in the polyester Vestopal W (Ryter and Kellenberger 1958), 6 in the polyester Vinox K 5 (Kellenberger *et al.* 1956) and 2 in methacrylate (Newman, Borysko and Swerdlow 1949). Paraffin embedding was done for optical controls.

Most of the ultra-thin sections were cut using a Servall's Porter-Blum Ultramicrotome fitted with glass knives; a few were later obtained with an LKB Ultratome. Sections showing interference colours of silver and straw-yellow were collected over 20% alcohol or acetone or simply over distilled water. Only very rarely was chloroform vapour employed to stretch them (Sotelo 1957). Sections were carried over supporting grids previously coated with a thin film of formvar. Observations were made with an RCA (type EMU 5C) electron microscope, operated at 50 kV and equipped with an objective aperture of diameter 50 μ . Micrographs were usually taken at initial magnifications ranging from 2,500 to 25,000 diameters.

In order to facilitate understanding of the spacial disposition of structures in the epidermis over larger areas, many survey pictures were taken at low as well as high magnifications which were later mounted together. About 250 such montages have been made.

III. Observations on Normal Tissue

In classical cytology, the epidermis of the amphibians is generally considered as composed of three layers: a corneal layer formed of thin flattened cells, an intermediate layer beneath, and finally a basal layer often called the germinal layer or the layer of Malpighi. In the newt *Triturus cristatus* Laur., the thickness of the epidermis of the caudal region is usually made up of a minimum of three or four cells, increasing in number at places. Excepting the corneal layer, the tissue shows little differentiation into distinct strata, unlike in the mammalian counterpart. In the semi-fine sections of the polyester-embedded tissue used for optical controls as well as in the low magnification electron-micrographs of the "montages", the general morphology is well recognised. The corneal layer by its characteristic deep staining is easily distinguishable in optical preparations. Sometimes a doubling of this layer is seen at places. The first row of cells lying underneath the corneal layer may appear very different from one preparation to another, showing presumably transitional stages between the typical cells of the inter-

mediate layer beneath and the corneal cells above. These are referred to here as subcorneal. The bulk of the epidermal tissue is formed of cells lying between the subcorneal layer and the basal one. These cells are termed "typical" here for convenience of description; they constitute the intermediate layer. At the base of the epidermis, the boundary-cells form the basal layer. This layer is sharply demarcated from the underlying dermal elements by a distinct adepidermal zone easily recognisable in electron micrographs. As is well known in classical cytology (Rabl 1951), the epidermis also includes a variety of other cells, the descriptions of which do not conform to those of a typical representative from the layers mentioned above. Purely on the basis of the clearer appearance of their cytoplasm in the electron micrographs, these different kinds of cells are arbitrarily placed here in a group called "clear cells".

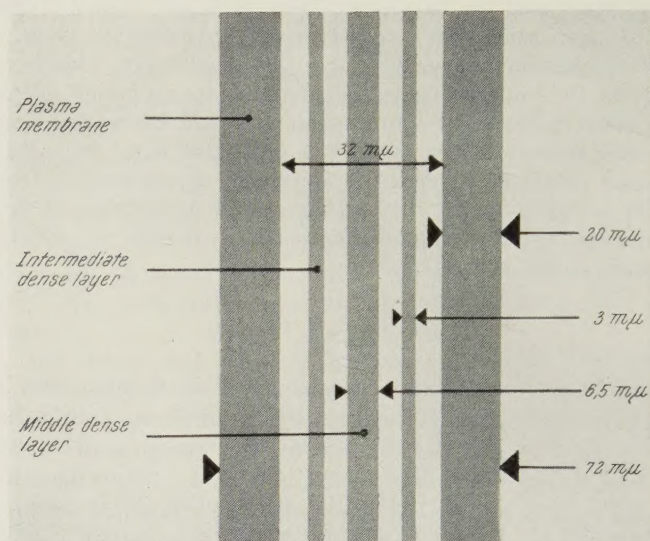
Corneal layer

The external surface of the corneal layer is thrown into many short and irregular processes, and is outlined by the uniformly thick (about $20\text{ m}\mu$) plasma membrane which characterise the component cells (Pillai, Guénin and Gautier 1960, Figs. 5 to 9). Sometimes a thin, dark layer (about $5\text{ m}\mu$) is noticeable especially on this outer surface; it runs a parallel course to the above-described plasma membrane, and is separated from it by a light layer of about $8\text{ m}\mu$. The significance of the fine structure of the membrane systems revealed by electron microscopy is a topic lively discussed among biophysicists (Robertson 1959; Schmitt 1960). To enter into the conflicting views regarding this problem is outside the scope of the present work. Concerning the plasma membrane and the desmosomes, the fine structures are only described as observed in high-resolution electron micrographs which exclude possibilities of gross artifacts.

Laterally, the adjacent cells of the corneal layer are closely apposed to each other with much diminution of intercellular spaces and comparatively great extension of desmosomal figures. The narrowest space between adjacent cells is usually found for a short distance towards the external surface (Pillai, Guénin and Gautier 1960, Figs. 5 and 7). The length of desmosomal apposition seems augmented in proportion to the reduction in amount of the intercellular spaces. Though the cells are greatly flattened, due to the lateral appositions being slant, the attachment areas are extensive.

The desmosomes in well-resolved high-magnification pictures show outside the plasma membranes, three dense and four light layers in alternate order. These recall comparable observations of other workers in different materials (e. g., in epidermis: Horstmann and Knoop 1958; Odland 1958; Hibbs and Clark 1959; in cervical epithelium: Vogel 1960; Karrer 1960 a; in buccal mucosa: Themann 1958; Faske and Themann 1959; in muscles: Sjöstrand, Andersson-Cedergrén and Dewey 1958; Karrer 1960 b). Often the middle layer is thicker than the intermediate ones (Plate 1, Fig. 2; Text-Fig. 1). Sometimes only a single middle dense layer is seen giving a superficial resemblance to the

"quintuple-layered" figure recently described by Karrer (1960 a and b) in different tissues.



Text-figure 1. Corneal desmosome: Diagrammatic representation of a section perpendicular to the plane of apposition. The measurements indicated were made on high-resolution electron micrographs.

The inner or lower aspect of the corneal layer is more even than the outer surface, and is separated from the cells occurring beneath by extensive subcorneal spaces surrounding sparse desmosomal attachments (Pillai, Guénin and Gautier 1960, Figs. 5, 6 and 9). As regards the

Plate 1 (Figs. 1—7). Corneal, subcorneal and intermediate layers.

(All the figures presented here are electron micrographs taken of material fixed in buffered Osmium tetroxide and embedded in polyester.)

Fig. 1. Corneal lining of a glandular duct in tangential section. $\times 50,000$.

Fig. 2. Desmosome between two corneal cells. $\times 280,000$.

Fig. 3. Desmosome between a corneal cell and a subcorneal one. Note the difference in thickness between the intermediate dense layers and the middle layer in Figs. 2 and 3. $\times 280,000$.

Fig. 4. Apposition between two subcorneal cells towards the subcorneal space. $\times 26,500$.

Fig. 5. Desmosome between two subcorneal cells. Note that only the intermediate dense layers are resolved. $\times 100,000$.

Fig. 6. Desmosome between two typical cells. No intradesmosomal dense layers are seen. In both the Figs. 5 and 6, the plasma membrane is seen as a triple-structure. $\times 100,000$.

Fig. 7. "Myelin-figure-like-body," or knot-like structure in a "normal" typical cell. $\times 50,000$.

Cr. C.: Corneal cell; D.: Desmosome; Sc. C.: Subcorneal cell; Sc. S.: Subcorneal space; T. C.: Typical cell.

Corrigenda: In Fig. 4 bottom and Fig. 5 top for "ScS" read "ScC".

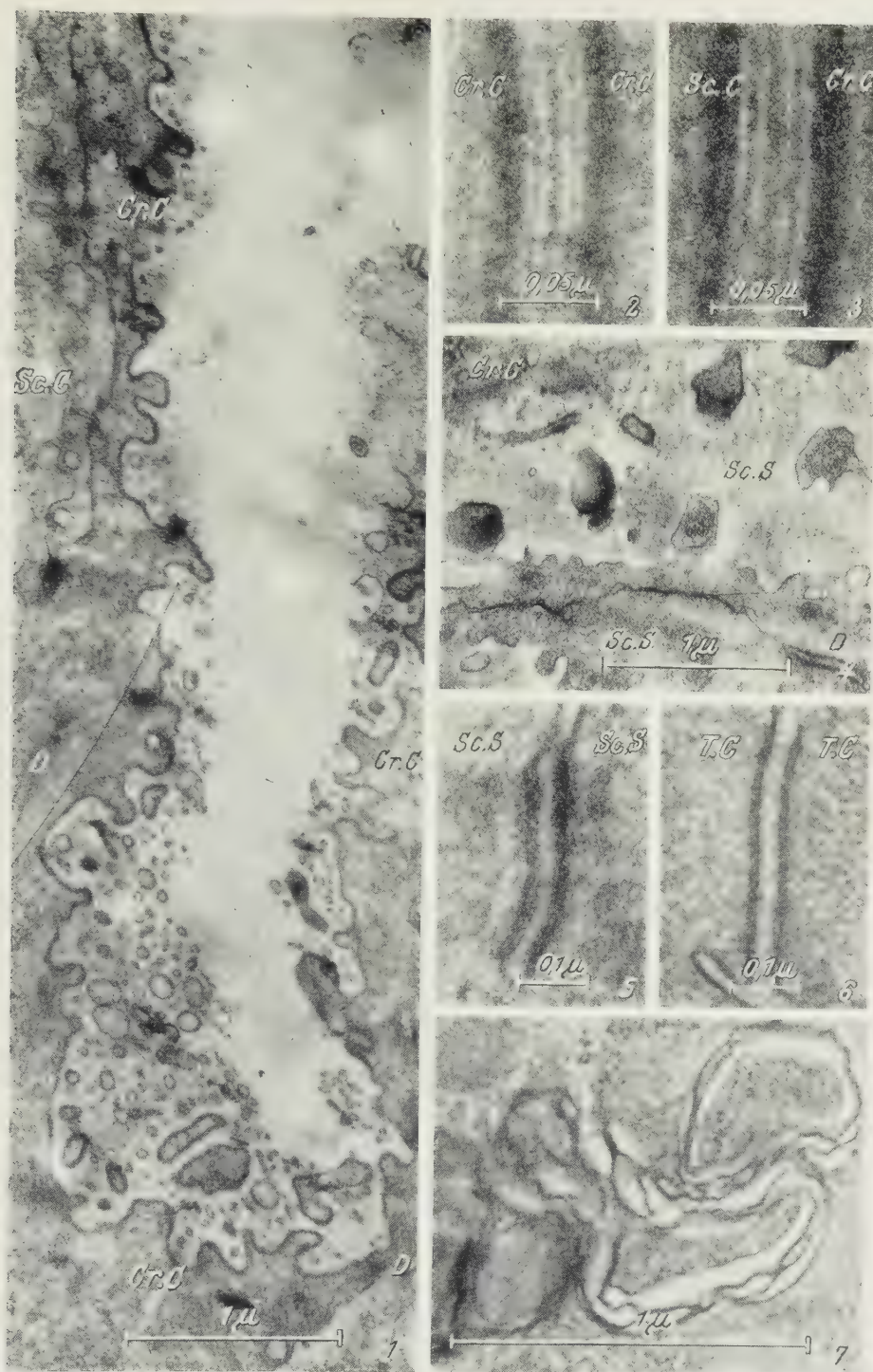
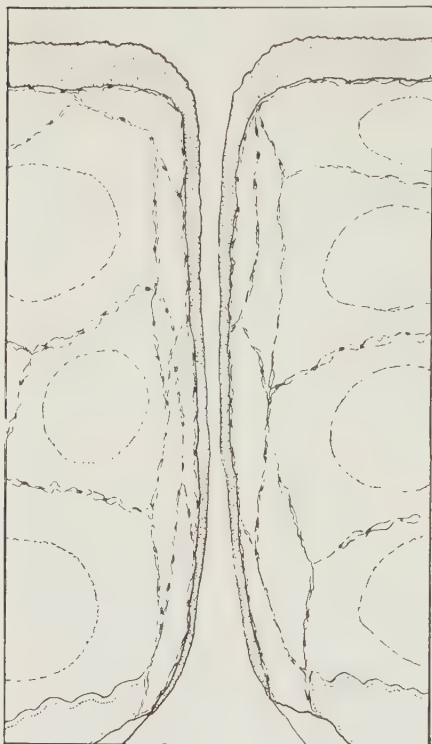


Plate 1 (Figs. 1-7).

structure of the desmosomes here, the following points may be noted: their disposition is roughly parallel to the surface. In fine structure, they resemble those described above (Plate 1, compare Figs. 2 and 3). The desmosomal moieties of the corneal and subcorneal cells differ in certain respects: usually, the former are formed of shorter protrusions than the latter; the bundles of tonofilaments which terminate at the apposing membranes are often very conspicuous in the subcorneal half, whereas such a structure is usually not represented in the corneal moiety. Thin protoplasmic processes jutting from the immediate neighbourhood of desmosomal appositions which characterise so often the attachment areas between typical cells are absent here; however, the subcorneal moiety may show short protuberances probably representing vestiges of the same structural entity.



Text-figure 2. Corneal layer: Schematic drawing of a transverse section of the epidermis passing through the lumen of a glandular duct. Note the difference in thickness of the corneal layer as it passes inwards.

The cytoplasm is mainly occupied by thick bundles of tonofilaments. In this feature, the corneal cells make a striking contrast to the typical cells. The individual filaments also appear slightly stouter here than in the latter and measure about $10\text{ m}\mu$ in thickness. These facts are all the more interesting when compared to that reported by Hibbs and Clark (1959) in the human epidermis. They state that "tonofilaments are evident in the cells of all layers except in the stratum corneum." Within the network of tonofilaments, mitochondria are often encountered distinguished by their double-lined contour and cristae. Usually, they appear spherical, possibly indicating a low activity. Certain dense bodies and vacuoles, all suggestive of degeneration, are also seen in the cytoplasm. Occasionally few

pigment granules may occur in these cells. No clear sign of Golgi bodies or ergastoplasm has been observed.

The nucleus is flattened. The nuclear membrane is either disrupted or absent (Pillai, Guénin and Gautier 1960, Fig. 6). The nuclear material appears granular and usually shows a dense and homogeneous distribution; however, there may occur irregular areas of lighter density and vacuolisation.

The corneal layer continues into the ducts of the cutaneous glands of

epidermal origin, forming a complete lining (Plate 1, Fig. 1 and Text-fig. 2). The layer is thinner here than usual, measuring at places only about $0.1\ \mu$.

Subcorneal layer

The subcorneal cells make contacts with the corneal ones through desmosomes as described in the previous section (Plate 1, Fig. 3). The space that exists between the two layers is termed here the subcorneal space. Some of the subcorneal cells show a complicated aggregation of vacuoles towards this space. Often, the relationship between these cells shows three different aspects: starting from the subcorneal space downwards, there is first a region of very close apposition with only less than $10\ m\mu$ between the plasma membranes of the contiguous cells (Plate 1, Fig. 4). This resembles the attachment between corneal cells towards the surface of the epidermis; but whereas in the corneal layer the corresponding portion is more straight (Pillai, Guénin and Gautier 1960, Fig. 7), in the present instance it usually shows a more or less rugged course. Secondly, following the area of close apposition, there is a region where many desmosomes occur close to each other giving a striking resemblance to the figure of apposition in the mammalian epidermis. Finally, in the lower part, the cellular relationship resembles that between typical cells of the intermediate layer. At the ultrastructural level, the desmosomes between subcorneal cells show faint indications of intradesmosomal dense layers (Plate 1, Fig. 5). On the whole, as regards cellular appositions, this layer shows in different preparations what could be termed transitional stages between those observed in the intermediate layer and the corneal one. The usual cytoplasmic organelles such as mitochondria, Golgi bodies, ergastoplasm, and microbodies are all notable in these cells; however, they may also show signs of atrophy or degenerative transformations. Tonofilaments show an increase in development; in some instances, they approach a very close resemblance to those in the corneal cells, thus taking again a transitional position between the typical and the corneal cells.

Certain large cells characterised by their clearer cytoplasm are often found in the subcorneal layer. These cells which present a special type of desmosomes are described below as the first type of "clear cells."

Intermediate layer

All the cells found between the subcorneal layer and the basal layer are included under this heading. Electron microscopically, the majority of cells look alike: they are called "typical cells." Marked differences are shown by certain cells occupying an intercellular position amongst the typical cells. These also being distinguished by a lighter shade of the cytoplasm, are placed under the arbitrary class of "clear cells."

Typical cells. These are attached to one another leaving large intercellular spaces around. These spaces appear as being formed by the sagging down of the plasma membranes from the desmosomal areas. Fine finger-shaped processes of the cellular margins, usually about 200 to $400\ m\mu$

long, may dangle in these spaces. This augments the cell surface considerably. The spaces are generally clear, but sometimes contain certain granular or filamentous material.

Each typical cell is outlined by a distinct plasma membrane, which may show variations in density. Its thickness is about $10\text{ m}\mu$. Except at the region of the desmosomes, it has not been possible to resolve it into finer sublayers. At the desmosomes, the plasma membrane seems rigid, denser, thicker and sometimes resolvable into two dark layers sandwiching a lighter one. Usually the portions of the membrane immediately around a desmosome either sag down forming a trough or are raised into pseudopodia-like processes.

Special mention may be made here of a peculiar type of vesiculation of the plasma membrane that is infrequently observed in the normal cells, this phenomenon being much augmented and more frequent in the "neoplastic" tissues. These vesicles appear as bead-like profiles apparently replacing short lengths of the membrane. Such vesicles are present in the cytoplasm also in the vicinity of the plasma membrane showing such formations. These differ from the usual type of pinocytotic vesicles (Palade 1956; Schulz 1959; Campiche 1960; Pillai, Guénin and Gautier 1960) in being localised to areas where the cell membrane appears disrupted.

Desmosomes are formed of complimentary structures from contiguous cells and constitute a regular feature of a typical epidermal cell. They occur all over the surface of the cell, spaced very irregularly. Desmosomes, though are points of firm attachment between contiguous cells, are not places of closest approach of the neighbouring cells. Often in the immediate surroundings of a desmosome, a jutting process from one cell may show a closer proximity to the plasma membrane of the other. Sometimes such protoplasmic prolongations from the edges of the desmosomal areas may be seen on both sides either from the same cell (Pillai, Guénin and Gautier 1960, Fig. 4) or from opposite cells. These processes present different angular inclinations with the plane of apposition of the desmosomal moieties. The length of apposition of the desmosomal halves may vary much ($306 \pm 10\text{ m}\mu$, with a standard deviation of 127). On the contrary, the thickness of the plasma membrane ($21.8 \pm 0.6\text{ m}\mu$, with st.dev. 6.9) and the intradesmosomal space ($16 \pm 0.5\text{ m}\mu$, with st.dev. 5.9) show great constancy in measurements. Generally, desmosomes are formed at the meeting places of cellular processes. The plasma membrane at the apposing facets of the processes are thicker and denser. In each moiety of the desmosome, systems of tonofilaments extend from the plasma membrane towards the interior of the cell. However, it is worth mentioning that very often in the micrographs, the tonofilamentar bundles are better shown in one moiety than in the other, which difference seems not entirely due to the plane of sectioning. Sometimes bundles from neighbouring desmosomal halves in a cell may show apparent connections with one another. Often one or more striations parallel to the plasma membrane and transverse to the tonofilaments occur on the proximal side of a desmosomal half as

described by Porter (1956) and seen in the illustrations of Fawcett (1958, Fig. 34) and Pillai, Guénin and Gautier (1960, Fig. 1). The tonofilaments from each moiety make some sort of alignment with each other in the opposite side, though it has not been possible to ascertain whether the filaments cross the intradesmosomal space or not. However, often, faint lines suggesting a preferential orientation of the intervening substance has been noted (Pillai, Guénin and Gautier 1960, Fig. 4) as did Horstmann and Knoop (1958) earlier. It is of interest to recall here the suggestive diagram on cell contact given by P. Weiss (1958) recently. The cytoplasm of the two cells in the immediate vicinity of a desmosome presents a diffuse density which hinders a clear visualisation of the relationship between the tonofilaments and the plasma membrane. Selby's (1955) contention that the filaments terminate in granules has not been substantiated. Cross-sections of desmosomal moieties show sometimes a twisted disposition of the filaments in a bundle.

The exact nature of the intradesmosomal substance is not known. Different pictures have shown a fibrillar, granular or lamellar configuration. Faint lamellar figures comparable to those described in the corneal and subcorneal layers have been seen only very seldom. What seems certain is that the space is occupied by some kind of cement material which may be involved in holding together parts of contiguous cells.

Systems of tonofilaments are seen to traverse the cytoplasm in all directions. Often they run parallel to the surface of the cell seemingly connecting the different bundles from the desmosomes as has been mentioned above. Sometimes they follow the outline of the nucleus or encircle mitochondria. It is possible that they form a continuous elastic system contributing to the mechanical stability of the cell as suggested by Charles and Smiddy (1957). Tonofilaments tend to increase in quantity in the cells towards the corneal layer.

The ergastoplasm appears as a system of membrane-bounded closed spaces usually represented by more or less tortuous canals and vesicles of various sizes. On its outer aspect, the membrane bears many almost even-sized granules: the granules of Palade or ribosomes. These are also seen in abundance freely scattered outside the ergastoplasmic membranes. The outer envelope of the double-walled nuclear membrane, which may show connections with the ergastoplasmic elements, also bears the same type of granules. The interior of the ergastoplasmic saccules shows a density similar to that of the cytoplasmic ground substance.

A well developed Golgi apparatus is present. The constituents of this organelle appear as narrow canals and vesicles. Often these elements occur in two groups which tend to show a bilateral symmetry. These canals and vesicles seem to differ, as is well known, from those of the ergastoplasmic elements only in being devoid of the granules of Palade and in being more regular in width and extension. Sometimes, the Golgi bodies seem to take a symmetrical orientation in apparent relation to a desmosome (Pillai, Guénin and Gautier 1960, Fig. 5).

Mitochondria occur in large numbers and often in groups. They show

different shapes varying from long and filiform to short and rounded. The length, which has been ascertained from sections, vary from 0.5 to more than 1μ , whereas the width averages about 0.2 to 0.4μ . A mitochondrion is outlined by a double-walled envelope. The cristae usually have a transverse disposition to the long axis of the mitochondrion. Often several dark granules of about 30 to $40m\mu$ in diameter are present inside a mitochondrion.

Somewhat resembling mitochondria in form and size are certain lightly dense bodies scattered in the cytoplasm. These present a stiffer form which may be round or ovoid. These bodies, the significance of which is much discussed (Rhodin 1954; Rouiller 1960; Rouiller and Bernhard 1956), are referred to here as dense microbodies. They are outlined by a distinct membrane which is single and not double unlike that of mitochondria. The inside of these bodies usually appear uniformly granular. Sometimes seemingly transitory forms are encountered between them and mitochondria.

Some other structures are also found in the cytoplasm, but less frequently than those mentioned above. Such are the irregular dark bodies, small vacuoles, pigment granules and "myelin-figure-like bodies". The pigment granules have the same structure as those found in the chromatophore cells and are invested with a single membranous envelope. In only one specimen of "normal" epidermis certain bodies reminding of myelin figures have been observed (Plate 1, Fig. 7). These show a superficial resemblance to the knot-like structures described below in the NaCl-treated tissues. But, in the present instance, the abnormal membranous formations are often found around or in relation to abnormal mitochondria, whereas no such relations have been found in the case of the NaCl-treated tissues.

The nucleus is large and roughly spherical in shape. The surface contour is usually wavy and often one or more notches are seen which may

Plate 2 (Figs. 8—12). Clear cells and nerves.

Fig. 8. Part of a "clear cell with desmosomes" showing its peculiar intercellular relationship. Note the close apposition of the contiguous cell. $\times 20,000$.

Fig. 9. Part of a chromatophore cell showing characteristic pigment granules. At the left side of the picture, part of another clear cell is seen. $\times 19,000$.

Fig. 10. Part of a "clear cell with conspicuous inclusions." At the top of the picture, part of another clear cell can be seen. $\times 19,500$.

Fig. 11. A nerve fibre in the intercellular space showing a bulbous enlargement with many mitochondria and a longitudinal portion having double-lined strands inside. The tissue was treated with 5% Complexon solution. The mitochondria inside the nerve are apparently intact whereas in the epidermal cells they are much distorted. See for example, the mitochondrion seen in part at the top of the picture towards the right-hand side. $\times 43,000$.

Fig. 12. Part of a "clear cell without inclusions" surrounded by four epidermal (basal and typical) cells. Note the long and stout processes of the clear cell. $\times 19,500$.

B. C.: Basal cell; *Ch. C.*: Chromatophore cell; *CL. C.*: Clear cell; *D.*: Desmosome; *I. B.*: Inclusion body; *N.*: Nucleus; *P. G.*: Pigment granule; *Sc. C.*: Subcorneal cell; *T. C.*: Typical cell.

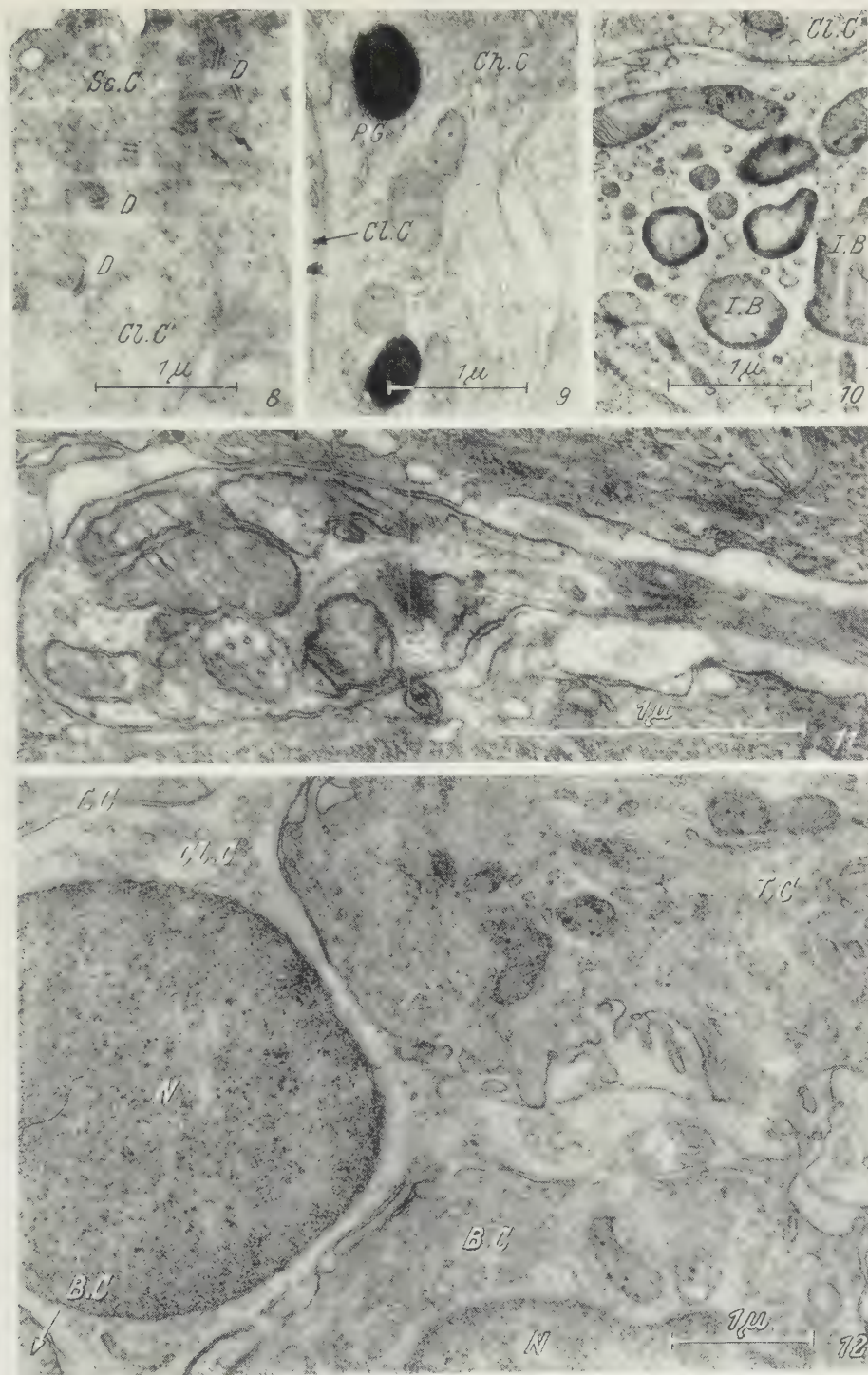


Plate 2 (Figs. 8-12).

traverse deep inwards. The nucleus is enveloped by a double-membrane which unite at places forming pores. Often a thin membrane-like structure may be seen to extend across these pores similar to that shown by Watson (1955). The external wall of the nuclear membrane may show, as mentioned above, connections with ergastoplasmic elements and also bear granules of Palade. The nuclear material appears granular. Two types of granules have been noted, a smaller and a larger one. The smaller ones resemble the ribosomes. The assembly of the larger granules usually give a rough reticular figure within the matrix formed by the smaller ones. Nucleolar profiles appear as dense granular conglomerations.

Clear cells. As mentioned above, certain cells are found among the subcorneal, intermediate and basal layers, which differ from the majority of their neighbours. These being easily spotted out by the clearer shade of the cytoplasm, are classified under the term "clear cells" (Plate 2). As often happens, when only small portions of these cells are encountered in the intercellular spaces, it is difficult to distinguish them one from the other. In order to classify these cells the following criteria are adopted: firstly, the mode of intercellular relationships—the presence or absence of desmosomes thus determining two main groups; secondly, the occurrence of particular cytoplasmic inclusions enables subdivision among the "desmosome-less" group.

The first group is constituted by large epidermal cells usually found in the subcorneal layer (Plate 2, Fig. 8). They form desmosomes with contiguous cells. The appositions, however, show certain peculiarities: the desmosomal moiety of the clear cell is usually formed of a depression instead of a protrusion from the cell; the intercellular spaces surrounding these cells are much reduced. The cytoplasm contains bundles of fine tonofilaments scattered all over. Other organelles are much less abundant than in the typical cells. The nucleus is large and the nuclear material may show differences in distribution: large, clearer areas with comparatively sparse granulation mark off dense regions in the middle as well as at the surface beneath the nuclear membrane.

The plasma membrane of the cells of the second group, being free from desmosomal appositions with contiguous cells, follows smoother courses. The pseudopodia are long and stout, but much less numerous compared to the small processes of the typical cells. Nuclei in comparison with that of the typical cells are usually more rounded and their granularity appears more uniform. Mitochondria are fewer and tonofilaments are almost absent. In this group, three kinds of cells are noted. Often two cells belonging to different kinds of this group are seen side by side or one almost completely encircling the other (Plate 2, Figs. 9 and 10).

The chromatophore cells bearing the characteristic pigment granules compose the first kind (Plate 2, Fig. 9). These cells are usually provided with long pseudopodial extensions which traverse the intercellular spaces in many directions. In thin sections, these extensions being usually crosscut are clearly distinguishable only if pigment granules are present. Each pigment granule is enveloped by a single membrane. Often around

this membrane, scattered black particles are seen which are considered as products of reduction of the osmium tetroxide. The interior of these granules is occupied by a material showing great electron-density; except for faint indications of an irregular scale-like lamination, this material lacks any other definable structures. These pigment granules differ from the melanin granules of the human epidermis by the presence of a well defined external membrane and absence of clear internal laminations (Charles and Ingram 1959) or any regular reticulum (Drochmans 1960). The mitochondria in the chromatophore cells are usually larger and stouter than those of the typical cells, but contain similar dense granules inside. Ergastoplasmic elements are seen in abundance.

The clear cells without desmosomes constituting another kind occur among or near the basal cells, but are never seen contributing to the formation of an adepidermal complex. They are bulky cells which usually do not show long pseudopodia-like extensions and are characterised by conspicuous inclusions in the cytoplasm (Plate 2, Fig. 10). These inclusion-bodies are often much larger than the mitochondria and may be of many shapes: e.g., round, oval, irregularly stellate. Some of these inclusions show a concentric lamellation especially on their cortical portion. Dense microbodies, similar to the ones described in the typical cells, are also encountered. Mitochondria are few, but do not show any special features.

Finally, the desmosome-less clear cells forming the third kind are distinguished by the absence of any particular cytoplasmic inclusions (Plate 2, Fig. 12). They show long and stout pseudopodia-like extensions which wind along the intercellular spaces of other cells. They seem to be rather of soft consistency being pushed or constricted by the small protrusions of neighbouring typical cells. The clear cytoplasm contains numerous vacuoles or vesicles and few free Palade-granules. Mitochondria are only rarely seen. As the cell is pressed against by other cells, the nucleus is often elongated; in cross-section it shows a small diameter (2 to 4μ), but in a favourable longitudinal section it measures about 16μ . A typical nuclear membrane is present enclosing a granular mass.

Nerve fibres. It is very difficult to distinguish a nerve fibre transversely cut, from small portions of the different kinds of clear cells. Infrequently nerve fibres appear as longitudinal sections, then they are easily recognised on the basis of the features recently described by Hay (1960), in regenerating salamander limbs: bulbous enlargements containing numerous small mitochondria, small vesicles and longitudinal extensions with elongate tubules in a clear cytoplasm.

Basal layer

The cells of this layer while resembling the typical ones of the intermediate layer, differ from the latter in showing fewer and apparently labile desmosomes and modifications of the plasma membrane at the adepidermal zone. The desmosomes are usually shorter in the length of

apposition and are seemingly disposed to easy breakage. The intradesmosomal space appears clearer than that between typical cells. The space is usually wider and often the opposing plasma membranes of the desmosome deviate from a parallel course to a diverging one. The intradesmosomal space often shows faint fibrillar connections between the apposing plasma membranes; but excepting a faint indication once, the intradesmosomal lamellar figures are not observed. The plasma membrane (Plate 3) facing the dermis shows, at irregular intervals, peculiar dense, thick spots: these recall the "bobbins" of Weiss and Ferris (1954 a, b, and 1956), although they differ from the latter in fine structure; these differences may well be the result of different techniques employed or due to the differences in the developmental stages of the tissues studied. Bundles of tonofilaments may be seen converging towards these spots. Sometimes a doubling of the plasma membrane has been seen along the entire lower margin of a cell including the spots just described (Plate 3, Fig. 13). Incidentally, it may be remarked here that an apparently similar structural differentiation could be seen in a micrograph of rat's lingual mucosa published by Palade (1958, Fig. 139), though not described as such. Another feature of the plasma membrane facing the dermis is the pinocytotic activity evidenced by the appearance of vesicles (Plate 3, Figs. 14, 16, and 17; Pillai, Guénin and Gautier 1960, Fig. 12).

Dermo-epidermal junction. The junction between the epidermis and the dermis is characterised by a clear space and a diffuse line in the electron micrographs; these two show a roughly parallel disposition to the plasma membrane of the basal cells facing the dermal side. The three structures together are considered as constituting an adepidermal complex, extending the terminology introduced by Salpeter and Singer (1959

Plate 3 (Figs. 13—17). Dermo-epidermal junction.

(All the figures in this plate have the epidermis shown on the left side, and dermis on the right.)

Fig. 13. The plasma membrane of the basal cell shows a triple-layered contour and irregular dense thickenings. $\times 42,500$.

Fig. 14. At arrows, fine filamentous strands are seen extending from the plasma membrane to the adepidermal membrane through the adepidermal space. $\times 49,000$.

Fig. 15. Note the termination of the collagenous fibrils of the adepidermal reticulum (basement lamella) near or into the adepidermal membrane. $\times 14,500$.

Fig. 16. The relations between the fibrils and the adepidermal membrane are shown at higher magnification. $\times 52,500$.

Fig. 17. At thick arrows, the adepidermal membrane is cut tangentially showing the diffusely granular nature of its substance. Note also the termination of the fibrils near the adepidermal membrane. Near the bottom, an apparent breakage is seen in the plasma membrane through which a diffuse substance seems to be extruding. $\times 52,200$.

Ad. M.: Adepidermal membrane; *Ad. S.*: Adepidermal space; *C. F.*: Collagenous fibrils; *D. T.*: Dense thickenings of the plasma membrane; *F. S.*: Filaments strands; *P. M.*: Plasma membrane; *P. V.*: Pinocytotic vesicles.

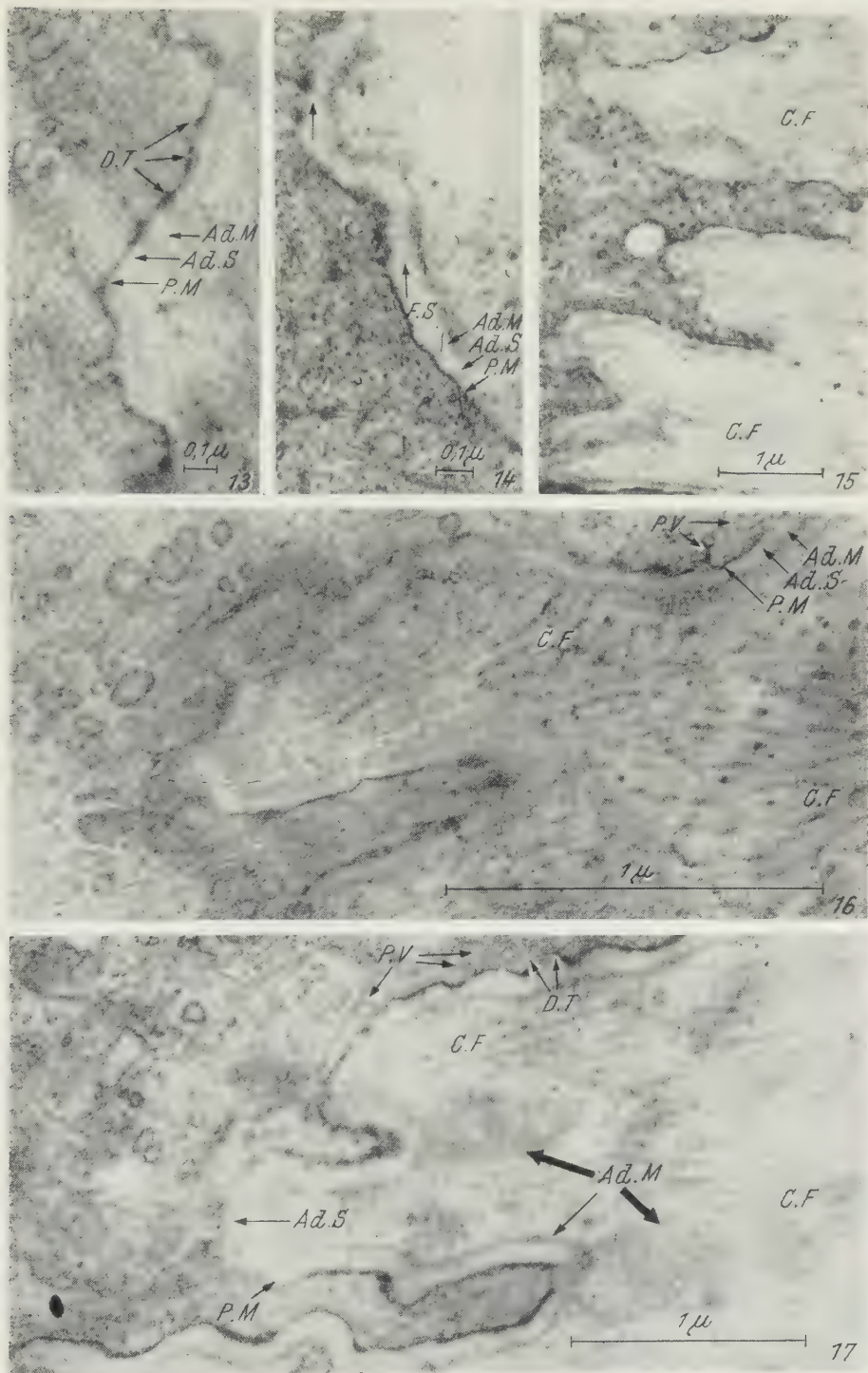


Plate 3 (Figs. 13-17).

and 1960). The clear space which may vary in diameter from about 50 to 80 m μ is called the adepidermal space and the structure represented by the diffuse line, the adepidermal membrane which varies in thickness from 15 to 20 m μ . This membrane usually presents a granular aspect (Plate 3, Figs. 13 to 17); sometimes it gives a cross-striated appearance. When cut tangentially (Plate 3, Fig. 17), it is usually found to be finely granular. Sometimes thin filamentous strands are seen to extend from the plasma membrane, traversing the adepidermal space (Plate 3, Fig. 14; Pillai, Guénin and Gautier 1960, Fig. 11), similar to those shown by Holmberg (1957) in the epithelium of the rabbit's eye.

The collagenous fibres of the dermis underlying the adepidermal membrane (constituting the "basement membrane" of the classical cytologists) sometimes show a close proximity to the latter. The fibres in such instances appear as either originating from or terminating at the adepidermal membrane (Plate 3, Figs. 15 to 17).

IV. Observations on Treated Tissues (Excluding "Neoplasia")

It was thought that observations on the ultrastructural alterations induced in the epidermal tissue by certain abnormal conditions may provide interesting comparison for the probable specific changes related to the development of hyperplasia following the administration of carcinogen. Thus brief observations were carried out on the effects of treatment with heterotonic media, a chelating agent and subcutaneous injection of pure ground-nut oil.

Effects of hypertonic media

In order to see whether a simple disturbance of the osmotic balance in the tissue will alter or break desmosomal connections, the effect of hypertonic solutions was tried at first. Treatments were performed both *in vivo* and *in vitro*.

In vivo. Tissues were excised from the tails of animals under complete anaesthesia and whose posterior regions up to the hind limbs were completely immersed in solutions of different tonicity for 30 minutes. Thus two animals were treated with 1%, three with 2% and three with 5% solutions. These eight samples of tissues were separately fixed and processed to provide materials for the following observations.

Plate 4 (Figs. 18—20). Treated tissues.

Fig. 18. Piece of tissue treated with 1% NaCl solution for 20 minutes. Knot-like structure formed in the cytoplasm of a typical cell. Note the continuity of the membrane of the "knot" with that of the ergastoplasm at arrows. $\times 54,000$.

Fig. 19. Buffer-treatment for 50 minutes. Note the fine granulation of the cytoplasm and the nucleus. Part of a clear cell is seen on the left-hand side in the intercellular space. $\times 8,000$.

Fig. 20. Extensive vacuolisation of the cytoplasm in the cells of an animal whose tail was immersed in 5% NaCl solution for 30 minutes. The vacuoles in this picture are indistinguishable from the intercellular spaces. $\times 26,500$.

CL. C.: Clear cell; D.: Desmosome; I. S.: Intercellular space; N.: Nucleus; V.: Vacuole.

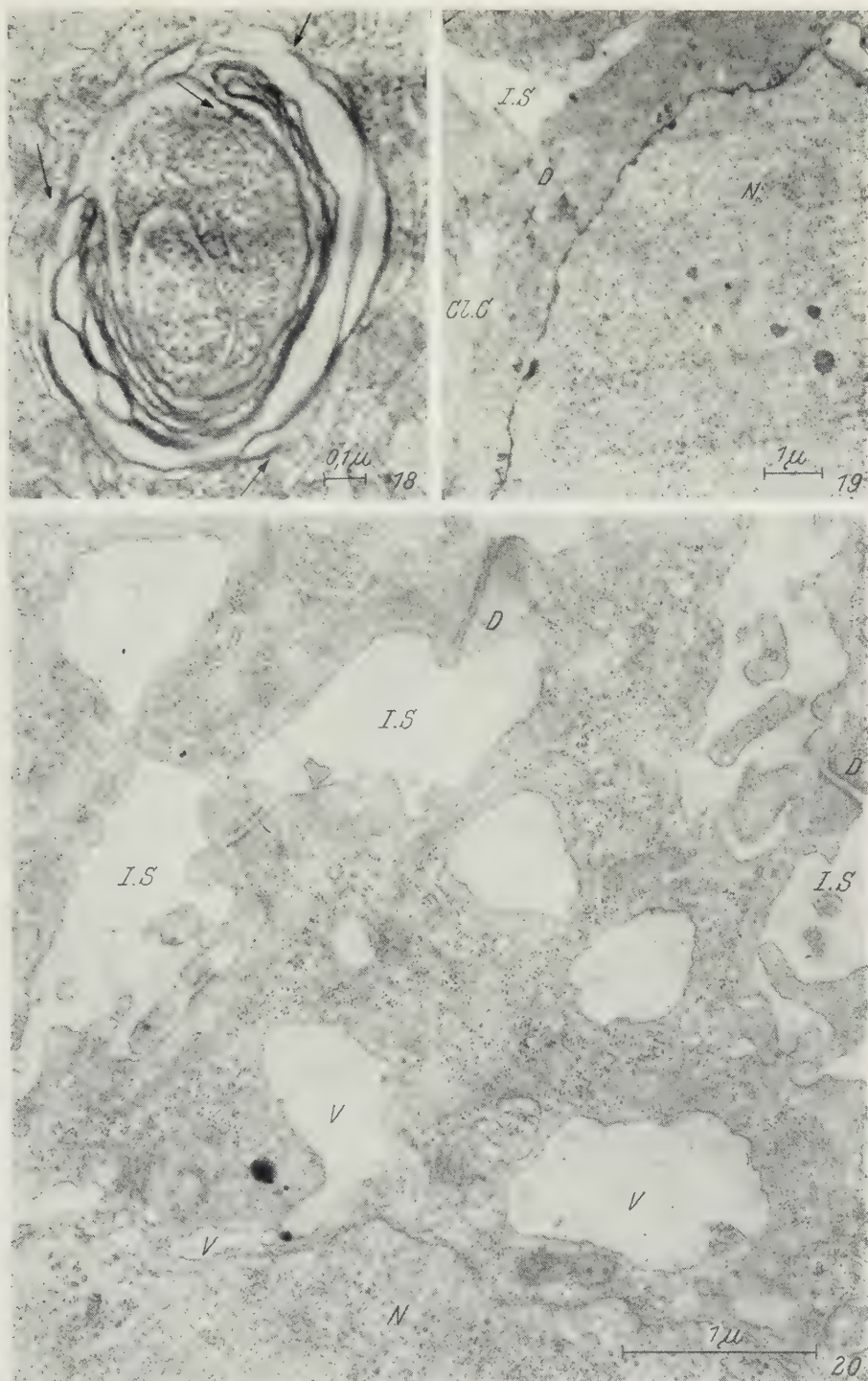


Plate 4 (Figs. 18—20).

In the animals treated with 1% NaCl solution, the intercellular spaces are found to be enlarged. The protoplasmic processes of the cell margins are increased in number, thus enhancing the area of the cell surface. The cytoplasm shows irregular fissure-like vacuolar formations edged by thick and dark membranes, the latter usually forming complicate knot-like structures (Plate 4, Fig. 18) resembling myelin figures in degenerating cells. These abnormal membranes may be continuous with the ergastoplasmic elements as seen in the above figure. However, they do not bear the granules of Palade, though the latter may be found in great numbers in close assembly outside the "knots". In the mitochondria, sometimes the usual dense granules are not observed; at other times they appear to have definitely increased in number, a single mitochondrial profile showing up to 16 granules in one instance. Thus though the intra-mitochondrial granules seem to be affected by the salt solution, no precise relationship has been observed as did J. M. Weiss (1955) in the duodenal absorptive cells of mice fed on large amounts of sodium and potassium salts.

In the animals treated with 2% solution, the vacuolisation of the cytoplasm seems to have increased. The vacuoles appear more swollen showing rounded outlines in section. In some of these vacuoles detached membranes apparently forming entire capsules are seen. These membranes are often double, each constituent being as thick as the plasma membrane.

Vacuolisation of the cytoplasm attained huge proportions in the animals treated with 5% NaCl solution (Plate 4, Fig. 20), which is easily observable in optical sections. They are often found disposed around the nucleus. Sometimes they appear even larger than the nuclei, pressing the latter out of shape and pushing them to one side or other of the cells. Detached membranous capsules of various sizes may be seen in these vacuoles as described above. Sometimes the nuclear material shows irregular precipitations.

In vitro. Tissues were taken from animals under complete anaesthesia and immersed immediately into the solutions of different tonicity. Five samples were studied. Of these, the first two were treated with 1% NaCl solution for 20 minutes before fixation. No differences worth mentioning

Plate 5 (Figs. 21—23). Treatment with distilled water.

Fig. 21. Parts of two cells showing signs of swelling and degeneration. Arrows indicate nuclear pores. $\times 21,000$.

Fig. 22. Desmosome between two typical cells shown at high magnification. Note signs of a lamellar figure in the intradesmosomal region. Portions of unidentified cells are seen on the right-hand side of the picture. $\times 97,000$.

Fig. 23. Parts of three cells illustrating the action of distilled water on epidermal cells. Note the cellular contour which lacks the characteristic finger-like processes. Desmosomes apparently remain intact. The intercellular spaces contain granules which look like the ribosomes in the cytoplasm. Cytoplasm and nucleus show fine granularity. Mitochondria and ergastoplasmic sacs are swelled up. $\times 16,000$.

D.: Desmosome; *E. R.*: Ergastoplasm; *I. S.*: Intercellular space; *M.*: Mitochondrion; *N.*: Nucleus.

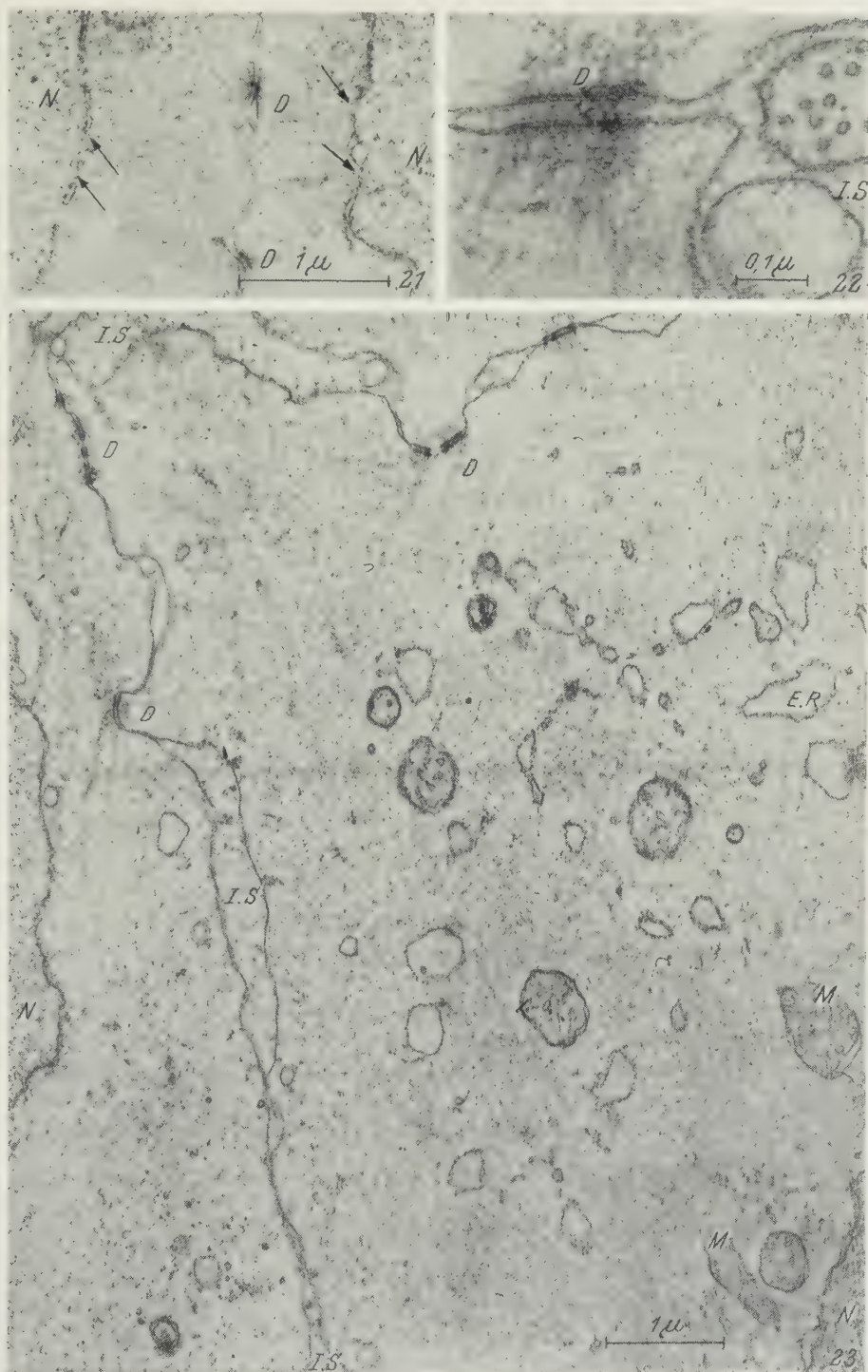


Plate 5 (Figs. 21–25).

were noted between these tissues and those taken from animals whose tails were immersed in 1% salt solution for 30 minutes. The three remaining samples were treated, each one with a solution of different strength (1%, 2% and 5%), all of which being adjusted to a pH of about 7.4. All these tissues show similar changes as those treated *in vivo* except the one treated with 5% solution; in the latter, the *in vitro* treatment has produced much less of the perinuclear vacuolisation which was so conspicuous in the *in vivo* treatment. As regards the knot-like structures, in the present instance they appear darker and often their membranous components unite to form thick and more regular bands which give a superficial resemblance to the myelin sheath of nerve fibres. In the adepidermal complex, small vacuoles are formed by the retraction of the plasma membrane at places between the dense thickenings, similar to the observations illustrated by P. Weiss (1958, Fig. 8).

The most important fact as regards the purpose with which these observations were carried out is that no appreciable changes have been noted concerning the structure of the desmosomes (Plate 4, Fig. 20).

Effects of isotonic medium

0.8% NaCl solution at pH 7.4 was used to serve as a nearly isotonic solution. A piece of skin scraped from an anaesthetised animal was immersed in this solution for 30 minutes before fixation. Electron micrographs obtained of this material look much like that of the normal tissues.

Effects of hypotonic media

Thinking that observations on the effects of a different balanced medium may serve as a further control, veronal acetate solution (0.076 M) at pH 7.4 was used to treat a freshly excised piece of epidermal tissue. This piece was fixed after 30 minutes of immersion in the buffer solution.

A general swelling of the cellular elements is evident in almost all micrographs (Plate 4, Fig. 19), including the optical ones. Sometimes the intercellular spaces appear reduced whereas at other times they look more or less normal. Portions of clear cells occurring in the intercellular spaces are identified as such in some cases; in others since only the membranous envelopes are distinctly seen, identification becomes difficult. Desmosomes seem to show no change at all.

Inside the cell, ergastoplasmic elements are swollen as also the mitochondria. Mitochondrial cristae are usually distorted. Cytoplasm as a whole appear more evenly granular than the normal. No change has been noted as regards the tonofilaments. Nuclei are more rounded and their substance shows more or less uniform granularity (Plate 4, Fig. 19). Small dense accumulations, often attached to the inner aspect of the nuclear membrane may represent the nucleolar materials; they show an enhanced contrast here than in the micrographs of normal cells.

As a further step in the series of heterotonic media, pure distilled water was chosen. The materials for this study were obtained from a non-

anaesthetised animal. An excised piece was immersed in distilled water for 50 minutes before fixation, and cut into smaller ones inside the fixative.

The tissue as a whole appeared swelled up (Plate 5), this being far greater than that observed in the case of treatment with the buffer solution; the swelling is quite evident in the optical micrographs also. Cellular outlines become less folded with consequent reduction and modification of intercellular spaces and effacement of the usual small finger-like processes. Sometimes the intercellular spaces show fine granular accumulations in them which apparently resemble the granules of Palade in the cytoplasm (Plate 5, Fig. 23). With all these changes in the intercellular region, the desmosomes appeared still intact. However, it is interesting to note that the intradesmosomal material has shown some signs of a lamellar configuration between typical cells (Plate 5, Fig. 22); such resolution having not been possible in the untreated material, may favour the view of an alteration of the cement substance.

The cytoplasm appears finely granular (Plate 5, Figs. 21, 23), there being a remarkable augmentation in this feature here over the buffer-treated tissue. The ergastoplasmic vesicles are much distended, but the granules of Palade are still seen attached to them on their outer sides. Mitochondria are swollen; the envelope and cristae are distorted; the mitochondrial granules seem less contrastive than in the normal cells. The dense microbodies are also swollen and sometimes their membranous envelopes may be broken. Tonofilaments are traceable in the cytoplasm as more discrete bundles, this being most clear in the proximity of the desmosomes.

The nuclear outline appears irregularly wavy and roughly spherical. No deep notches which often characterise the normal nucleus are observed. Usually the double walled nuclear envelope does not show evidences of much modification. However, in a few instances, large perinuclear spaces have been seen which seem to be the result of an extreme swelling up of the space enclosed by the two nuclear membranes. In such cases, some nuclear material also has been observed in the form of spherules in the spaces, probably a stage in the disintegration of the nucleus. Usually the nuclear material appears finely and evenly granular which, however, show greater density of distribution compared to the cytoplasmic granulation mentioned above. Sometimes these granules may seem to emerge through apparently disrupted nuclear pores (Plate 5, Fig. 21).

Parts of clear cells occurring in the intercellular spaces have been found difficult to distinguish one from the other.

Effects of chelating agent

Electron microscopic observations of Coman (1954) on liver cells subjected to the action of a chelating agent have shown marked retraction of the apposing cell surfaces. This positive result was used to support a theoretical consideration (Pillai 1957) that the desmosomal attachments may be mediated through the presence of calcium ions. In order to test this hypothesis, the chelating agent Complexon III (a product of Siegfried

A. G., Zofingen, Switzerland) was employed in aqueous solutions of different strength. Two preparations were made from freshly amputated tails immersed in 1% solution for 5 and 10 minutes respectively. Two other preparations were made from excised pieces of skin treated in 1% solution for 10 and 20 minutes before fixation. Preparations were further made of two tissues treated in 2% solution for 15 and 20 minutes respectively; and two others treated in 5% solution, one for 10 minutes and the other for 15 minutes. Of these, the 5% solution is nearly isotonic.

No remarkable differences from the normal tissue were observed in the first two preparations which were treated in 1% solution for 5 and 10 minutes. In the case of excised pieces treated in 1% solution, intercellular spaces are reduced as a result of slight swelling of the cells. Mitochondria and ergastoplasmic vesicles also show signs of swelling.

Intercellular spaces are very much reduced in the tissues treated with 2% solutions (Plate 6, Fig. 27). Plasma membranes of contiguous cells run parallel to each other with less intercellular space than exists at the desmosomes. The latter do not show any discernible change. Mitochondrial envelope and cristae are distorted as a result of further swelling.

A general disturbance in all the membranous components of the cell is the most marked effect of a 5% Complexon solution. Intercellular outlines are so much distorted that they are difficult to follow. Cytoplasm is riddled with vacuoles with the result that it is not easy to make out the ergastoplasmic and Golgi elements. Mitochondria appear blown out of shape with the cristae breaking down and disappearing. However, these organelles are comparatively much less affected in the case of a supposed nerve fibre (Plate 2, Fig. 11). The outline of the nucleus is clear, but its substance

Plate 6 (Figs. 24—28). Treated tissues.

(Figs. 24 to 26 show effects of treatment with 5% Complexon solutions. In these three figures the position of the epidermis is up and that of dermis down.)

Fig. 24. Part of a basal cell protruding into the dermis. Note the scanty granulation in this part. Adepidermal membrane is not clear at the tip of the process.
×30,000.

Fig. 25. Part of a basal cell showing a breaking away of the cytoplasm from the plasma membrane which is held fast behind. Cytoplasm is much vacuolated.
×18,500.

Fig. 26. Part of a basal cell showing great vacuolisation. A disintegrating mitochondrion can be seen at the top. The adepidermal membrane is difficult to make out.
×14,000.

Fig. 27. Treatment with 2% Complexon solution. Parts of two typical cells showing an extreme reduction of intercellular spaces between. ×18,000.

Fig. 28. Tissue from oil-injected animal. Parts of two basal cells with large intercellular space. ×26,000.

Ad. C.: Adepidermal complex; *Ad. M.*: Adepidermal membrane; *Ad. S.*: Adepidermal space; *C. F.*: Collagenous fibrils; *D.*: Desmosome; *I. S.*: Intercellular space; *M.*: Mitochondrion; *N.*: Nucleus; *P. M.*: Plasma membrane.

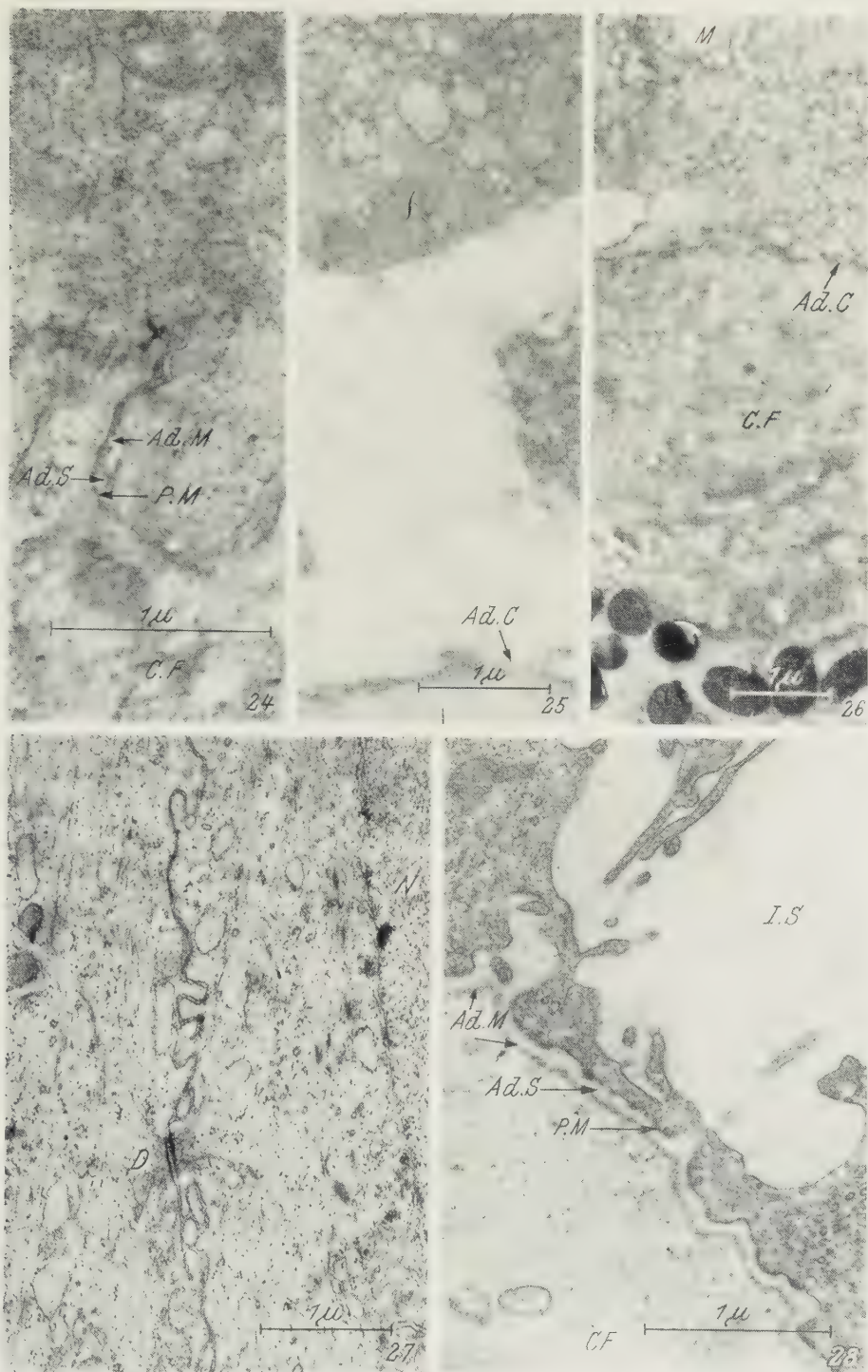


Plate 6 (Figs. 24-28).

loses the usual appearance being irregularly precipitated against a uniformly light background.

The most remarkable changes are to be noted in the basal cells along the adepidermal complex. In some of these cells, the downward processes dipping into the dermis are particularly swollen showing a hyaline texture and only few granular or fibrillar material inside (Plate 6, Fig. 24). In one case, a large portion of the cytoplasm is seen to be completely broken off from the plasma membrane beneath (Plate 6, Fig. 25). This picture shows a striking resemblance to one of P. Weiss (1958, Fig. 6) of amphibian basal epidermal cell detached from the basement lamella after an one-hour treatment with salivary amylase. Though usually the adepidermal complex is traceable, sometimes no sign of an adepidermal membrane is seen at places (Plate 6, Fig. 26). In such instances some granular material may be seen in the adepidermal zone.

Finally, it needs particular mention here of the negative result obtained as regards the action of the chelating agent on the desmosomes. They are apparently left intact. This may indicate that the desmosomes are more organically cemented structures than ones resulting through a simple adhesion brought about by calcium ions only.

Effects of injection of oil

Since the carcinogen methylcholanthrene used to provoke epidermal neoplasia was administered as a solution in pure ground-nut oil, the reaction of the oil alone was studied as a control for the interpretation of the effects of the carcinogen. The same quantity of oil as that of the solution of the carcinogen, was introduced in the same manner at the same site. Excision of the tissue was also performed in the same way. Thus two preparations were made from animals injected with the oil six days before and two from animals injected three days before.

The cells showed no notable cytoplasmic changes from the normal, except perhaps in the augmentation in number of the dark bodies in the tissues excised 3 days after the oil-injection. But these bodies, it should be noted, are small and quite insignificant compared to those in the "neoplastic" material. The noteworthy effect shown very clearly in the electron micrographs, is the enlargement of the intercellular spaces. This is especially remarkable towards the basal layer, where often only thin protoplasmic extensions could be seen over the adepidermal zone (Plate 6, Fig. 28). Sometimes intercellular spaces are riddled with protoplasmic extensions from contiguous cells.

No alterations in the structure of the desmosomes have been noted.

V. Observations on "Neoplastic" Tissues

The materials for the following observations were obtained from animals injected subcutaneously with 0.2 cc. of 2% or .5% methylcholanthrene in pure ground-nut oil, 15 preparations from the same number of animals were studied, of which 10 were made on the 6th or 7th day after the injection of the carcinogenic solution and 5 on the 12th day.

Since the neoplasia does not clearly show itself outwardly, excision of exclusively neoplastic material was extremely difficult. So tissues obtained from or near the neoplasia were used for observations in the electron microscope. When some of these tissues showed remarkable differences in fine structure, these differences themselves have been taken as criteria to decide whether the particular tissue examined was neoplastic or not. The following structural features were observed in the tissues excised on the 6th day after the injection, i.e. when the neoplasia is considered to attain an optimum development (Plate 7 and 8).

Alterations of ordinary structures

Though not a specific character shown by the "neoplastic" tissues only, pronounced enlargement of the intercellular spaces is notable in all "neoplastic" materials examined. These spaces are especially large towards the basal layer and are easily recognisable in optical sections. They are very conspicuous in electron micrographs as shown previously (Pillai and Gautier 1960, Figs. 5 and 6).

No marked differences are noted in the intercellular relationships between the corneal cells. But a great increase in the subcorneal space occurs sometimes. In the intermediate layer, the cells seem to assume more spherical shapes (Plate 8, Fig. 34; Pillai and Gautier 1960, Fig. 2). Though no differences are observed as regards the fine structure of the desmosomes (Plate 7, Fig. 30), the diminution in number of these contact areas is quite apparent. The processes of the contiguous cells which form desmosomes are often thinner and longer as the spaces between the cells are enlarged. The enhancement of the intercellular spaces is greatest between basal cells as mentioned above. Here, where the attenuated processes of the cells support large intercellular spaces, sometimes degenerating and disintegrating cells are encountered. Often certain dark type of cells (Plate 7, Fig. 31) occur adjacent to the degenerating ones, the former presumably in a state of vigorous growth. These two extreme types at once recall to mind the cell types A and B distinguished by Caspersson (1950) on the basis of functional activity for protein synthesis among cancer cells by microspectrographic studies. Recently such types of cells have been described by Haguenau (1959) in the human mammary glands. The rupture of the dermo-epidermal junction is so easily observable in optical preparations that it is taken as a criterion for the neoplastic reaction. However, an exact localisation of this is so difficult that it has not been possible to get that region in thin sections in any of the materials examined here.

In addition to the incidental changes following the enhancement of the intercellular spaces, the plasma membranes show an augmentation of the peculiar vesiculation described in the normal tissues. These vesicles may attain a diameter of about 100 $m\mu$ and appear generally in a chain-like configuration. The plasma membrane in the neighbourhood of such vesicles often shows intermittent faint areas giving an impression of breakages (Plate 7, Fig. 32).

Mitochondria look normal except in the degenerating cells, where they may be swollen and the cristae show various stages of disintegration.

However, the usual dense granules described in the normal cells are only rarely seen; they are almost altogether absent in the cells that show the peculiar structures to be described below.

In certain cells, the Golgi apparatus seems abnormal in being distorted and the vesicles being filled with a semi-dense non-granular material (Plate 7, Fig. 35). These abnormal type of Golgi elements are sometimes seen in close proximity to the light areas and lamelliform structures to be described below.

In one tissue, a peculiar phenomenon of membrane-infoldings has been noted which is especially well marked in the basal cells. (Plate 7, Fig. 29). These formations seem to compartmentalise the cytoplasm. The double-membranes of these infoldings are frequently seen to break up into small vesicles which dot the course for short distances. The intercellular appositions amongst such cells are quite indistinguishable from these membrane-infoldings except for the occurrence of desmosomes and parts of clear cells in between. Intercellular spaces are reduced to a minimum.

Appearance of "new formations"

Three different structures which seem to be intimately related, make their appearance in several of the "neoplastic" materials studied. They are tentatively described as "dark bodies," "light areas" and "lamelliform structures."

Similar when small to the spontaneously occurring dark bodies in the normal tissues and that mentioned in the case of oil-treated animals, the "dark bodies" present very conspicuous inclusions in the "neoplastic" tissues (Plate 7, Fig. 32; Plate 8, Figs. 34, 36, and 37; Pillai and Gautier 1960, Figs. 2, 4, and 6). They occur in all layers of the epidermis and often in

Plate 7 (Figs. 29—35). "Neoplastic" tissue.

Fig. 29. Membrane-infolding and vesiculation (arrows), attaining its extreme development in the basal cells in one preparation. It is difficult to distinguish between intercellular outline and true infoldings. $\times 29,000$.

Fig. 30. Two intact desmosomes from the intermediate layer. $\times 50,000$.

Fig. 31. Cellular alterations in the basal layer. Parts of adjacent cells showing extremely different aspects. The swollen appearance of the left one is presumed to indicate degeneration, while the dense cytoplasm of the right cell is considered to signify active growth. $\times 17,600$.

Fig. 32. Peculiar membrane-vesiculation seen in two contiguous cells. Note the disrupted-appearance of the plasma membrane near the area of vesiculation (arrows). $\times 27,500$.

Fig. 35. Abnormal Golgy body. The vesicles are filled with a lightly dense homogeneous material. Note proximity to light-areas. $\times 42,000$.

Ad. M.: Adepidermal membrane; *Ad. S.*: Adepidermal space; *D. B.*: Dark-body; *E. R.*: Ergastoplasm; *G. B.*: Golgi body; *I. S.*: Intercellular space; *L. A.*: Light-area; *M.*: Mitochondrion; *P. M.*: Plasma membrane.

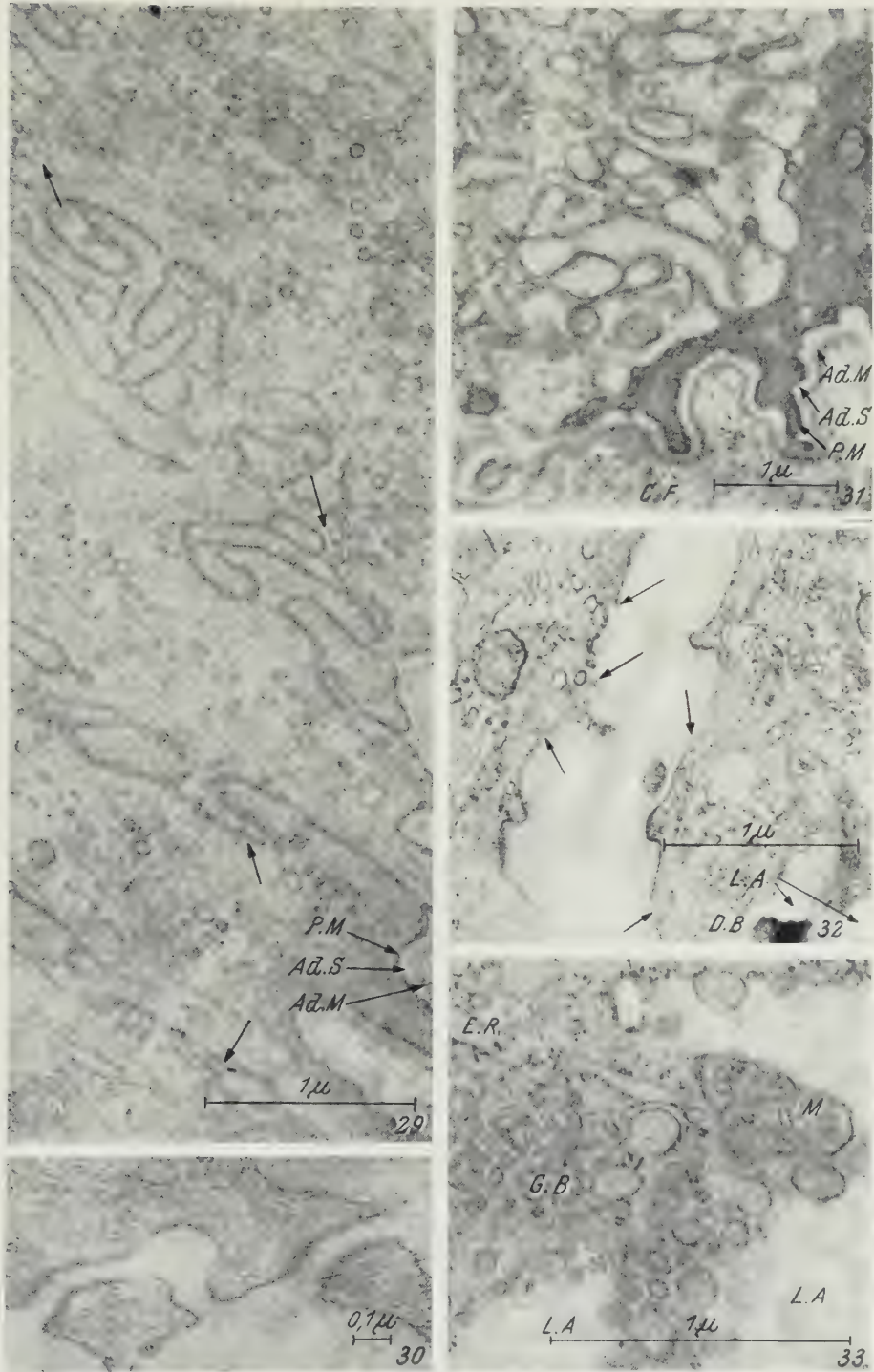


Plate 7 (Figs. 29-33).

groups. Their sizes vary from fractions of a micron to several microns in diameter. They are roughly, roundish bodies usually having an irregular and ill-defined outline. The banded appearance they take in sections is evidently produced by the vibrations of the microtome while sectioning. Granules of Palade are usually seen in considerable numbers in the neighbourhood of these bodies and sometimes actually merging with them. It is not certain whether the dark masses encountered in the dermis in optical micrographs and supposed to be oil-accumulations are related to these dark bodies seen in the electron micrographs.

The term "light areas" is used to describe the surprisingly clear areas found in many cells of the "neoplastic" materials (Plate 7, Fig. 35; Plate 8, Figs. 34, 35 and 37). In general, they appear lighter than the cytoplasmic matrix and the intercellular spaces. They even present a clearer shade than the blank areas of pure polyester in the sections outside the tissue-materials. However, these areas are not completely blank; they usually contain some amorphous, foggy material which occasionally present a granular aspect. Amidst the amorphous material there may occur few organelles such as mitochondria or bundles of tonofilaments. The light areas are not limited by any distinct barrier; the usual cell constituents are seen around them apparently pushed back.

The "lamelliform structures" (Plate 8, Figs. 34 and 36; Pillai and Gautier 1960, Figs. 2 and 4) appear generally inside the light areas as if being spun out of the amorphous mass inside. But they are not always strictly confined within the light areas; sometimes a lamelliform structure may more or less completely fill or even delimit a light area, when normal cytoplasmic structures are seen contiguous or continuous with the outer lamella. At times, they may show intimate contacts with the dark bodies; they are even found completely encircling the latter. Most impressive is the disposition of the lamelliform structures: at low magnifications, they resemble a system of parallel lamellae formed around a centre, a dark body for instance; at higher magnifications, these lamellae look like flattened

Plate 8 (Figs. 34—37). "Neoplastic" tissue.

Fig. 34. Parts of three cells with large intercellular spaces between. Desmosomes are reduced in number. Large light-areas and groups of dark-bodies occur in the cytoplasm. Note the clearer appearance of the light-area compared to the intercellular spaces. $\times 9,500$.

Fig. 35. Parts of two cells showing intact desmosomes. Note a few granules of Palade in the light-area near the top of the picture. $\times 28,000$.

Fig. 36. Occurrence of lamelliform-structures and dark-bodies near the nucleus of a typical cell. $\times 22,000$.

Fig. 37. Part of a dark-body surrounded by light-area; granules of Palade are seen around. $\times 42,000$.

D.: Desmosome; *D.B.*: Dark-body; *E.R.*: Ergastoplasm; *G.B.*: Golgi body; *I.S.*: Intercellular space; *L.A.*: Light-area; *L.S.*: Lamelliform-structure; *M.*: Mitochondrion; *N.*: Nucleus; *Pl.G.*: Granule of Palade.

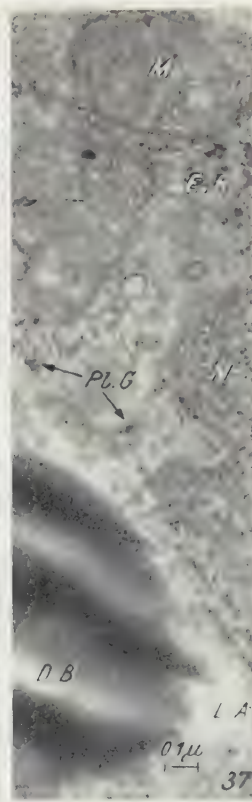
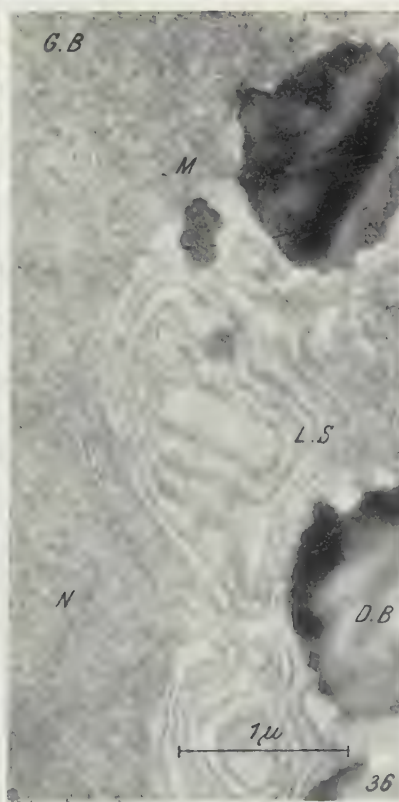
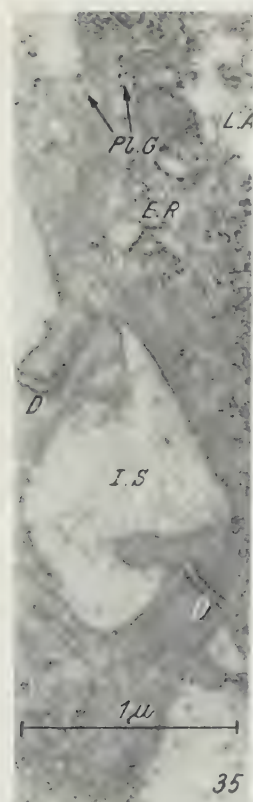
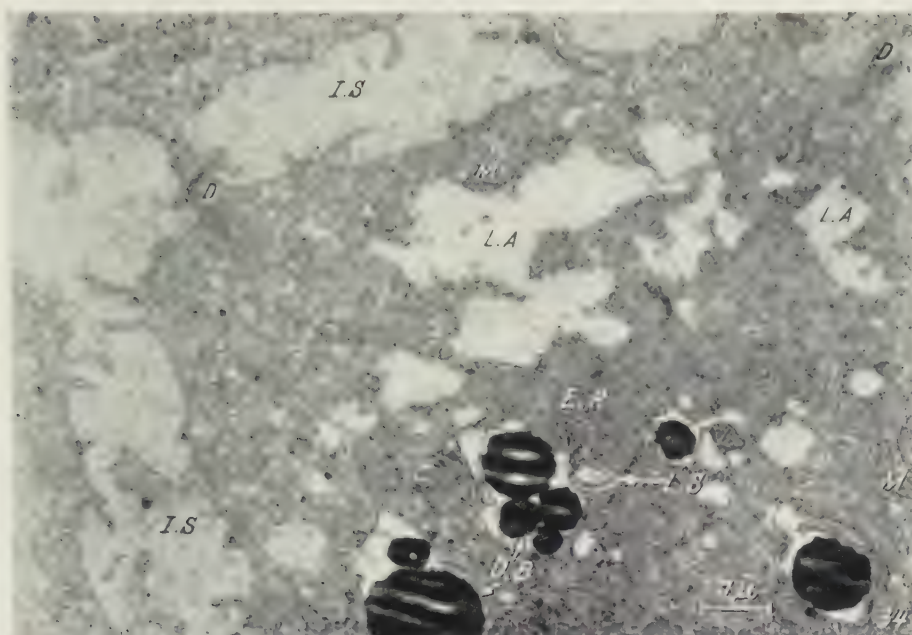


Plate 8 (Figs. 34—37).

bags. In fine structure, they resemble the smooth reticulum of Palade and Porter (1954). Chains or groups of bead-like profiles appear in connection with the lamelliform structures: this may produce an image of coalescence of the vesicles to form elongated saccules or conversely, of disruption of the latter into chains or groups of vesicles. The flattened membranous bags occur one over the other to produce the characteristic lamellated figure in sections. Often 4 to 6 membranous capsules may be seen in concentric disposition: in one instance up to 9 such lamellae have been observed. The components of these lamellae look essentially the same as the ergastoplasmic vesicles; at places it is possible to trace continuity between them. The most important difference between the two, however, is that the granules of Palade are generally absent in the case of the lamelliform structures except at times over the outer lamella.

In the five preparations made 12 days after the injection of the carcinogen, dark bodies are noted less frequently. Light areas and lamelliform structures are not seen. On the contrary, the peculiar vesiculation of the plasma membrane is seen much augmented. The desmosomes even in the areas showing much membrane-vesiculation appear totally intact.

VI. Comments on the Structure of the Epidermis

Corneal layer

The corneal layer forms an entire coat to the external surface of the animal. Even in 1876, Leidig considered it as a cuticle, though its cellular nature was repeatedly pointed out by Schulze (1867, 1869). Schulze's remarks on the strong lateral connections between the flattened cells of the corneum as the reason for the cohesion of the moulting layer is well borne out by the present observations. The long desmosomal attachments on the lateral sides, with short and sparsely distributed ones which join the layer as a whole to the subjacent cells, explain also why the corneal layer is so often seen peeled off from the underlying epidermal mass in optical as well as electron microscopic preparations.

The transformation of a typical epidermal cell into a corneal one involves, among other changes, a marked and apparently uniform thickening of the plasma membrane. This thickening appears to be of the same order as that of the desmosomal membranes in general. This modification of the plasma membrane may point to the following facts: 1. prolonged attachments between corneal cells, 2. desmosomes could only be formed at special modified regions of the plasma membrane, which are much limited in the typical cells and 3. attachment between the corneal and subcorneal cells are limited to the extent of membrane-modifications in the subcorneal cells.

Measurements have shown that the thickness of the plasma membranes approach uniformity at desmosomes amongst corneal and typical cells, whereas the intradesmosomal space is about double in the case of the corneal desmosome. It is worth remembering here that the one or three dense layers in the intradesmosomal space are very clear and easy to resolve in between corneal cells or between corneal cells and subcorneal ones, while

it is not possible to obtain such a picture in between typical cells. This favours the view of a qualitative change in the desmosomes as cells move on to take a superficial position. A comparable progressive increase in the complexity of desmosomal structures has been shown by Horstmann and Knoop (1958) in the epidermis of the rat.

Though the corneal cells show signs of degeneration, they do not seem to be dead altogether. A few intact mitochondria are often seen with well defined envelopes and cristae; however, as they always appear more or less spherical, they may well be considered as rather inactive. The occurrence of dark bodies in this layer of animals injected with the carcinogen may also indicate that the cells here are still capable of some metabolic activity. The high development of tonofilaments in the cytoplasm may contribute to the toughness of the layer forming the superficial coat. In the epidermis of the newt, development of tonofilaments is enhanced towards the corneal layer. This, as has been pointed out before, is quite different from what has been noted in the human epidermis by Hibbs and Clark (1959).

No notable changes in this layer have been observed in the tissues treated with heterotonic solutions or chelating agent, which indicate the rather impervious nature of the plasma membrane of the cells of this layer.

Intermediate layer

This layer may be compared to the *stratum spinosum* of the mammalian epidermis. The cytoplasmic organelles here seem in general better developed than in the mammalian prickly cells. The difference seems most marked in the case of the Golgi apparatus: while this structure is well developed in all the cells of the newt's epidermis excepting the corneal layer, it is seldom observed in the human tissue (Clark and Hibbs 1958). As regards desmosomes, the intradesmosomal dense layers are apparently less developed here than in the mammalian counterpart (Odland 1958, Hibbs and Clark 1959). However, an interesting similarity to be noted is this: the desmosomes between typical cells of the epidermis of the newt appear stronger than those that occur between basal cells: such a difference has been observed by Hibbs and Clark (1959) concerning the desmosomal attachments among the spinous cells and the basal cells in the human epidermis.

Desmosomes. A brief review of the history of our knowledge of intercellular attachments is recently given by Wood (1959) who describes for the first time the fine structure of the type of desmosome occurring in the epithelial cells of the coelenterate *Hydra*.

Electron microscopy has now confirmed the remarkable interpretation of Bizzozero (1864, 1870). It has also shown that the general pattern of desmosomal attachment is shown by different tissues, though these may differ in details of fine structure (e.g., in epidermis: Porter 1956; Selby 1955; Horstmann and Knoop 1958; Odland 1958; Hibbs and Clark 1959; in corneal epithelium: Sheldon and Zetterqvist 1956; in buccal mucosa: Sognnaes and

Albright 1956; Pillai 1957; Themann 1958; Fasske and Themann 1959; in cervical epithelium: Vogel 1960; Karrer 1960 a; in intestinal epithelium: Zetterqvist 1956; in muscles: Sjöstrand, Andersson-Cedergren and Dewey 1958; Fawcett and Selby 1958; Fawcett 1958; Karrer 1960 b; in blastomeres of sea-urchin: Balinsky 1959; in earthworm's nerve sheath: Hama 1959; in sea-anemone: Grimstone, Horne, Pantin and Robson 1958; in *Hydra*: Wood 1959). The desmosomes in the epidermis, cervical epithelium and buccal mucosa appear very much alike. However, further studies are needed for a correct understanding of the interrelationship of the various fine layers that are now discriminated. For Odland (1958) the "attachement plaques" are modified parts of the cell membrane and the "intermediate dense layers" are external structures. Working on the cervical epithelium, Karrer (1960 a) demonstrates that the intermediate dense layer of Odland is in fact the superficial part of the plasma membrane. According to Karrer, the dense zones called attachment plaques by Odland are mainly contributed by a "condensation" of the cytoplasm. These two views seem to be partially reconciled by the present observations on the desmosomes of the newt's corneal layer. Here, the plasma membrane shows a uniform density and thickness throughout, which corresponds to that of the desmosomal membranes elsewhere in the same epidermal tissue; no special condensation of the cytoplasm is observed. Further, in a few instances, the whole plasma membrane is seen to be invested with a fine superficial component which in the desmosomal regions seems to form the intermediate dense layer.

Though a molecular picture of the desmosome is still far from view, the available biological facts and theories may be put together to arrive at certain fruitful conclusions:

1. Desmosomes are specific contact areas between contiguous cells of same nature: in the epidermis, no desmosomes are formed between the various kinds of clear cells treated above as the second type; the clear cells with desmosomes are to be considered only as modified cells of epidermal origin. The term contact is used here as recently specified by P. Weiss (1958): "We shall consider a cell in contact with another body not only if the two surfaces are in direct apposition, but also if they are separated by a narrow space occupied by a molecular population whose free mobility is restrained."

The occurrence of the same type of structure in such different tissues as stratified epithelia on the one hand and musculature of the circulatory system on the other is most interesting. It points to analogous functions. The relations of tonofilaments in the one and myofilaments in the other appears highly suggestive.

2. Desmosomes are never formed between two processes of the same cell. Whatever may be the basis of this morphological rule, it definitely points to specific intercellular reactions for the development of these structures. An exception noted in a specialised case is that of the Schwann cell membranes enveloping the giant nerve fibres of the earthworm: here, "desmosomes" are formed between portions of the same cell encircling itself (Hama 1959).

3. The plasma membranes of the desmosomal areas are thicker, denser and appear more rigid. It is probable that in life, these specialised parts may be in a perpetual gel state. Gelation of protoplasm is associated with contractile tension (Lewis 1951).

4. According to Lettré (1952), the cell membranes of "resting cells" are in a state of permanent contraction which needs more energy than the periodic contractions during division. Since in the epidermal cells, permanent contracted state seems to be the nature of desmosomes (other parts apparently show perpetual mobility), the energy needed to maintain these structures may be considerable.

5. If desmosomal attachments are reduced in amount, as may well happen during injuries, energy exchanges between contiguous cells may be directly affected. More energy could be utilized for individual metabolism, aided in this function by more intercellular spaces, more surface area and possibly more nutrient material in the ambient medium. Contact inhibitions being lessened, the cells may be forced to resort to division and continue to divide until adequate contact relations are established between contiguous cells which ensure optimum exchanges of energy necessary to keep the balance.

6. The forms and functions that cells acquire in a tissue may be correlated to the intercellular contacts they make with each other. Since the cells of the epidermis are in a constant state of flux, the basal cells multiply and the daughter-cells move upwards while the corneal cells peel off in periodic moults, the intercellular contacts also should undergo modification. This is exactly what has been observed.

Clear cells: The problem of the nature of strange cells among the ordinary ones in the epidermis of the amphibians has been a much discussed one (Rabl 1951). Certain cells with long and slender prolongations have been noted by early cytologists who doubted the epithelial nature of those cells. They have recognized two types among these: one, containing pigments and the other, without pigments. The latter was again subdivided according to the presence or absence of granules which may be basophilic or acidophilic in nature. The cells with granules were considered to have been probably derived from leukocytes. Those without granules are the cells of Langerhans. The present electron microscopic observations seem to conform well with these classical groupings. However, depending on the clearer shade of the cytoplasm of the cells in the electron micrographs, the classification followed here is purely morphological and brings together quite different kinds of cells. It is to be pointed out also that the term "clear cells" has already been used to denote only the clear cells of Mason (melanocytes or dendritic cells) in the electron microscopic study of the human material by Clark and Hibbs (1958).

The clear cells with desmosomes may represent the "flask cells" (Flaschenzellen) of the classical cytologists, which have been described in all the amphibians except the *Perennibranchiata* (Rabl 1951). It seems,

being provided with desmosomes, these cells also contribute to the formation of the corneal layer. However, the functional significance of their peculiar features is a problem for further enquiry.

The chromatophore cells being the counterpart of the clear cells of *Masson*, are treated here as the first kind of clear cells without desmosomes. Their identity with the pigment cells of the classical cytologists needs no stress. It is a well known fact that ordinary epidermal cells are incapable of elaborating pigment granules themselves; the granules they come to possess are derived from the chromatophore cells. The latter are supposed to inject the pigment granules into the epidermal cells by a process termed cytotrine secretion. *Charles and Ingram* (1959) present certain electron micrographs of human epidermis which give "strong evidence for such secretion." This process has not been observed in the present materials, though often small portions of the chromatophore cells are to be seen closely applied to the epidermal cells.

The second kind of desmosome-less clear cells is easily distinguishable by the peculiar inclusion bodies. They correspond to the cells with acidophilic or basophilic granules described by the optical cytologists who considered them as probable leukocytes. The electron microscopic observations presented here seem to support this view. Lymphocytes are known to exist in the epidermis (*Trowell* 1958), where some workers have even suggested their transformation into epithelial cells. Electron microscopy seems to rule out this possibility, the cells being devoid of tonofilaments and incapable of forming desmosomal appositions.

The third kind of clear cells without desmosomes seems to correspond to the cells of *Langerhans* of the classical cytologists. It is possible that these cells may be only chromatophore cells that have lost or not developed the pigment granules, analogous to the conditions described in the human material by *Clark and Hibbs* (1958) and *Charles and Ingram* (1959).

Nerve fibres: The difficulty of distinguishing small branches in cross section from parts of clear cells has been pointed out by *Odland* (1958) and *Clark and Hibbs* (1958) in the human material and gladly supported by *Charles and Ingram* (1959). At present there is only the work of *Hay* (1960) which describes the intraepidermal nerve fibres in electron micrographs; in the present work her descriptions are used as criteria to designate certain intercellular bodies as nerve processes.

Basal layer

Situated at the lower boundary of the epidermis and being directly concerned with exchange of materials with the tissue fluid of the dermis, this layer forms the important germinal stratum. Terminologically, basal layer is adequately descriptive, though sometimes it has been referred to as the mucous layer (*Despax* 1920 and 1923). This layer is the centre of mitotic activity. Consequently, the constituent cells are maintained in a perpetual youthful state; they come under the category of "vegetative intermitotics"

in Cowdry's (1953a) classification. Desmosomes are fewer, lesser in the extent of apposition and are liable to easy breakages; these features contribute to the dynamic nature of this layer. The clearer shade of the intradesmosomal space in electron micrographs may be a reflection of the immature development of these attachment areas.

A conspicuous difference of the basal layer of the newt's epidermis from that of the mammalian counterpart seem worth noting: in the mammalian epidermis, the melanocytes (clear cells) are usually found bulging downwards from the general level of the basal cells (Clark and Hibbs 1958; Charles and Ingram 1959; Drochmans 1960), whereas in the present instance, the clear cells have almost never been seen to contribute to the formation of the adepidermal complex.

Dermo-epidermal junction

Electron microscopically, this region is marked by a complex of three structures: the plasma membrane of the basal cells, a light zone called the adepidermal space and a diffuse border termed the adepidermal membrane. This ultrastructural characterization of the dermo-epidermal junction being necessarily of recent date, is different from the view held by classical cytologists. For the latter, the epidermis is joined to the dermis by means of a basement membrane composed of connective tissue material. This "basement membrane" is now referred to as the basement lamella in the larval skin of amphibians (Rosin 1946; Weiss and Ferris 1954a, b and 1956; Kemp 1959) and subepithelial reticulum (Ottoson *et al.*, 1955) or adepidermal reticulum (Singer and Andrews 1956; Salpeter and Singer 1959) in the adult tissue. A discussion of the basement lamella or adepidermal reticulum goes outside the domain of the present work.

Now to consider the constituents of the adepidermal complex: the plasma membrane shows dense thickenings at irregular intervals where tonofilaments may be seen to terminate in clusters. Portions between these modified regions show pinocytotic activity; the self same portions are seen to invaginate into the cytoplasm producing vacuolar spaces as a result of treatment with hypertonic solutions. It may be stressed here that marked pinocytotic activity in the whole of epidermis seems confined to this region. The importance of such a function (Bennett 1956) in the physiology of the epidermis needs much attention. Thick areas corresponding to the ones mentioned above have been described in the mammalian materials also (Selby 1955; Charles and Smiddy 1957; Odland 1958; Hibbs and Clark 1959; Hinglais-Guillaud 1959). In the larval skin of amphibians, Weiss and Ferris (1954a, b and 1956) described certain bobbin-shaped bodies which, though may correspond to the above-mentioned dense thickenings, are said to have a different structure. P. Weiss (1958) has further discussed the significance of these "bobbins" in his article on cell contact. Though Kemp (1959) also refers to these formations in his study of the development of the basement lamella of the larval anuran skin, further

work is needed to elucidate their fine structure as well as their modifications during ontogeny.

The structural entity referred to as the adepidermal space seems to be the least discussed in the literature. Though the term "space" is not quite a happy one, it is used here for simplicity's sake. It corresponds to the 500 Å-granule-bearing part described by Weiss and Ferris (1956) in the larval amphibians. Working on the mammalian skin, Selby (1955) presumes that this space may contain some amorphous material, probably polysaccharide, which aid in binding the basal cell membrane to the adepidermal membrane. Pease (1960) in a general consideration of basement membranes, calls it the "cement substance"; according to him, it may possibly be some protein, but more plausibly polysaccharide. Kemp (1959) refers to it as the "hyaline layer" in the anuran skin. It is clear from these views that our knowledge of the nature of this "space" is still only in the conjectural state.

The adepidermal membrane belongs to the class of basement or basal membranes which are of universal occurrence when epithelial structures come in contact with connective tissue (Pease 1960). But the term "basement membrane" has been already committed to denote a different entity (the basement lamella) in the cutaneous structure by classical cytologists. Ottoson *et al.* (1953) tried to correct the mistake by calling the submicroscopic membrane the basal membrane and the fibrillar connective tissue layer the subepithelial reticulum. To avoid all confusion, Selby (1955) proposed a new term "dermal membrane" for the fine subepithelial structure. However, as Salpeter and Singer (1959) have argued, the term dermal membrane seems inadequate since the membrane is found between the basement lamella and the epidermis. Being unambiguous and quite descriptive, the name adepidermal membrane proposed by them has been accepted in the present work.

In view of their more constant association with the epithelia than with connective tissue, Pease (1960) questions the traditional concept that the basement membranes (ade epithelial membranes) are connective tissue components. On the basis of certain observations on the fine structure of regenerating limbs in the newt, Salpeter and Singer (1960) presume that the adepidermal membrane is differentiated "from an accumulation of intercellular ground substance in the proximity to the plasma membrane of the basal epidermal cells". The nature of the ade epithelial membranes is far from clear. It is presumed to be largely composed of polysaccharides (Pease 1960). Whatever may be the chemical distinctions of this membrane and the adepidermal space, these together with the plasma membrane of the basal cells form a firm complex. This is of importance for a strong binding of the epidermal tissue with the dermis below to maintain the tegumental integrity. The observation reported above of a breaking away of the cytoplasm from the adepidermal complex in a tissue treated with chelating agent supports this view.

The diffuse appearance of the membrane in electron micrographs with its lack of any vesicular formations may suggest its function as a fine filter for the exchange of fluids between the dermis and the epidermis. It may also help to maintain the special fluid environment (Crowdry 1955a and

1955) required for the integrity of the tissue. Further this membrane has been considered as controlling the differences in electric potentials in the frog skin (Ottoson *et al.* 1955).

VII. Comments on Treated Tissues (Excluding "Neoplasia")

The various treatments carried out to give a broader basis to compare the effects of the carcinogen, have yielded results which are also interesting on their own accounts. The fact that isotonic sodium chloride solution has not produced any remarkable submicroscopical changes in the tissue within 30 minutes appears interesting, though only limited observations were undertaken. After treatment with 1% solution, the intercellular spaces have enlarged, which was the result expected. The augmentation of the protoplasmic processes seems to indicate an enhancement of surface activity. But what was quite unexpected was the formation of the cytoplasmic fissure-like vacuoles which increased with the concentration of the salt solution. It has been noted by cellular pathologists that not only hypotonic solutions, but iso- and even hypertonic solutions sometimes produce cytoplasmic vacuolisation (Cameron 1952). These observations are now well substantiated by electron microscopy. The formation of knot-like structures, their enhancement with increasing concentration of the solution and their peculiar myelin-like appearance, may be an indication of a process of lipid dissociation in the cytoplasm (lipophanerosis—Policard, Collet and Prégermain 1958). The treatments *in vitro* and *in vivo* gave much similar reactions with lower concentrations of the salt solution: but with 5% solution, the *in vivo* treatment produced greater deviation in the form and size of the vacuoles.

The experiments with hypertonic solutions have shown that the intercellular cohesion at the desmosomes seems to be far stronger than expected, to be broken by the pressure exerted by these solutions through the enlargement of the intercellular spaces. Another way of explaining it is that the desmosomes may not be brought to bear the pressure, the proximal portions of the protoplasmic processes taking the brunt and getting lengthened as a result.

The buffer solution being hypotonic has produced slight swelling. This as well as the distilled water, though have produced differences in the granularity of the cytoplasm and the nucleus, did not evoke any comparable changes that were shown by the "neoplastic" tissue. Even the degenerating and disintegrating cells noted in the latter showed extremely different appearances.

The results obtained from tissues treated with solutions of the chelating agent show that in the epidermis, the desmosomal attachments are more organically united structures than was previously supposed (Pillai 1957). This is quite in agreement with the results recently obtained by Easty and Muto (1960) with Versene in the liver cells. However, more refined experiments than the mere immersion of the piece of tissue in aqueous solutions of the chelating agent may be undertaken to verify the present conclusions.

Tissues excised from animals 6 days after the injection of pure ground-nut oil show only enhancement of the intercellular spaces. The occurrence of small dark bodies in the tissues excised 3 days after the oil-injection points to a slight change in the lipid metabolism of the cells. However, these tissues failed to show any other abnormality connected with these lipid droplets.

The action of these diverse treatments is most remarkable in one point that the desmosomes have not shown any submicroscopical change, except perhaps in the single instance of water-treatment where the intradesmosomal cement seems to have been slightly modified.

VIII. Comments on Neoplasia

"A hyperplastic tissue is one in which the number of cells is increased above that present before the hyperplasia set in" (C o w d r y 1955). What is it that disturbs the normal balance? One may answer that it is some kind of stimulation which altered the normal metabolism. It is well known in the case of mammalian epidermal carcinogenesis (C o w d r y 1953 b) that, depending on the nature and amount of stimuli and the strain of the animal, the normal tissue could be stimulated to hyperplastic development and further to cancer. In the epidermis of the newt, the same carcinogen (methylcholanthrene) evokes a different reaction if administered in a different manner. L e o n e (1957) has shown that subcutaneous implantation of crystals of pure methylcholanthrene produces multiple cancers which metastasise in different tissues of the body. We do not know exactly the biological bases of such different behaviours. Our ignorance is well pronounced by C o w d r y (1955) when he deals with the chemical properties of cancer cells: "It is certain that hormones, enzyme concentrations, nerve impulses and a multitude of various other intrinsic or extrinsic factors, can influence the rates of intracellular chemical processes; yet no accurate description of the mechanism by which such effects are mediated is available in even one instance in any type of living cells."

In the development of the neoplasia of the newt, the stimulus for the abnormal growth is provided by the carcinogenic solution. What is observed as neoplasia after a few days may well be the result of a long chain of reactions; this may involve severe injuries and death to many cells, the products of injury in turn inducing division and modifications of the surviving cells. The changes undergone by the extracellular components may also be of great significance. Particularly important here is the breakage of the basement membrane at several places which allow the vigorously multiplying basal cells to infiltrate into deeper tissues. According to N e u k o m m (1944), the reaction of the newt's epidermis to carcinogens shows the enhancement of two phenomena normally manifested by epidermal cells: proliferation and elaboration of proteolytic enzymes.

The augmentation of these two activities following the carcinogenic stimulus results in the production of neoplasia which "digests" the basement membrane and infiltrates downwards.

Turning to the structural modifications noted in the present work, the pronounced enlargement of the intercellular spaces may be considered first. Though this is not a specific character, for it is seen in control tissue from

animals injected with pure oil, in tissues treated with hypertonic solutions and in regenerating epidermis (unpublished observations), it is quite remarkable in electron micrographs of neoplasia and cancers (von Albertini 1953; von Albertini, Glatthaar and Vogel 1955; Pillai 1957; Glatthaar and Vogel 1959). Concurrently a diminution in number of desmosomes is also observed. This may mean a lessening of the normal mutual tension sustained by cells in the healthy tissue. Such a release from the pressure of integration may give the cells additional energy for individual metabolism. Large intercellular spaces may offer more nutrient materials to more extensive-surfaces of the cells. Greater assimilation, thus, coupled with lesser controlling influences through lessened contact pressure, may be an important factor which incite the cells in the disturbed area to multiply.

The enhanced vesiculation of the plasma membrane appears enigmatic. Is it a stage in the breaking down of cells or processes of cells? Sometimes chains of vesicles extending the whole thickness of protoplasmic processes may suggest a disruption of the latter forming desmosomes. It is of interest to recall here that though enlargement of intercellular spaces has been great, no changes in the fine structure of the desmosomes have been observed. In fact, the present work was started with the hope of finding qualitative modifications in desmosomes during the development of neoplasia. This hypothesis has not been substantiated by the present observations. It is of importance that further studies at evaluating differences in intercellular exchanges during the genesis of neoplasia should take this fact into consideration.

The reduction of intercellular spaces in one instance, where curious membrane-infoldings have been described, shows a superficial resemblance to that in the tissue treated with 2% Complexon. In the former instance, the phenomenon is considered to be due to the cellular pressure resulting from excessive multiplication in, or adjacent to, the neoplastic region. In the latter case, it is clearly due to the swelling of the cytoplasm.

As regards the appearance of "new formations" described in the cytoplasm of "neoplastic" tissues, their likeness to the pre-cancerous changes produced in the hepatic tissue of rats by two different carcinogens is of great interest. Porter and Bruni (1959) used the aminoazo dye 3'-Me-DAB, adding it to the food of animals kept on a control diet. Emmelot and Benedetti (1960) and Benedetti and Emmelot (1960) administered the carcinogen dimethylnitrosamine intravenously as an aqueous solution. However, as will be shown below, comparable structural modifications have been noted in a variety of other pathological conditions.

The conspicuous "dark bodies" observed in the "neoplastic" cells of the newt's epidermis may be considered to have been formed in two different ways. First it was thought that these bodies may be the product of assimilation of the oil-vehicle of the carcinogen. But since no such bodies or traces of them were seen in the intercellular spaces, this assumption seems unwarranted. A second way of interpreting the occurrence of these bodies

is to consider them as the result of an abnormal cellular reaction stimulated by the carcinogen. Since small dark bodies were seen in the normal tissues and the tissues from animals injected with only pure oil, the appearance of large bodies may be taken as the result of an augmentation of a normal process or its derangement. Porter and Bruni (1959) have noted a great increase in the accumulation of lipid droplets which was estimated to occupy about a quarter of the cell's volume. They consider it as a sign of disturbed lipid metabolism. Emmelot and Benedetti (1960) have observed the formation of dark bodies starting inside the ergastoplasmic sacs. Such a relation has not been noted in the present work, though the final products look very much alike. Advancing evidences from studies on cell fractionation, Emmelot and Benedetti support their electron microscopic observations for an increase in lipids in the liver cells after the administration of the carcinogen.

Amorphous dark bodies of similar appearance are generally considered as accumulation of lipids or phospholipids related to degeneration of the cell. Comparable structures have been described by several authors in induced pathological conditions: Mishra and Herman (1960) in necrotic cells of the heart muscles as a result of induced stress; Schmidt and Themann (1960) in the Yoshida tumour cells as a result of treatment with the cytostatic substance Endoxan. Thus the accumulation of lipid bodies in the cytoplasm may be taken to represent a definite disturbance of the normal lipid metabolism of the cells.

Seeing the first electron micrographs of the surprisingly light areas, the immediate reaction was to dispose them as artifacts. But when these were seen more or less consistently associated with equally abnormal structures, they necessitated approval as representing real structural alterations. Since in many micrographs they appear lighter than any region inside or outside the cytoplasm, the artifact produced by the destruction of some substance occupying the area may be considered possible: through inability of the OsO_4 to fix, or dissolution during preparation of the tissue for sectioning, or sublimation due to electron bombardment inside the microscope. Another view which, if confirmed, is of extreme interest is that these light areas may represent accumulation of some polysaccharide. Since the five years polyesters have been used as embedding medium, no other substance has shown a similar lack of density as the starch grains in plant tissues (Heitz 1957; Blondel and Turian 1960). But there the clear areas are completely blank whereas in the present instance, these regions contain a nebulous material which may sometimes show a granular aspect. The light areas in the "neoplastic" tissue may be compared to the areas of glycogen in the liver cells (e.g., Ham 1957), but since the embedding materials are different, caution should be taken in comparative interpretations.

Somewhat similar areas seen in different kinds of cells and all denoting degenerative changes could be mentioned here. In the Yoshida ascites tumor cells subjected to the action of a cytostatic agent, Schmidt and Themann (1960) have found necrotic changes existing together with a dissociation ("Entmischung")

of protoplasm. One of their pictures with a "light area" containing a few dark bodies shows some resemblance to those obtained of the "neoplastic" cells. Porter, Zahnd and Clavert (1960) working on hepatic cells of certain lower vertebrates under the action of folliculin, show extensive clear areas devoid of organelles, but it is difficult to make an exact comparison with their printed pictures. Mishra and Herman (1960) observed "areas free from any filaments" in lesions of cardiac muscle cells following induced stress. Taking into consideration all these facts, it is the opinion of the present author that the light areas represent most likely regions of degenerate metabolism.

The "lamelliform structures" may be compared to different kinds of organelles revealed by electron microscopy in different tissues under normal, experimental or pathological conditions, all having the common feature of a concentric disposition of the membranous systems. This comparison may aid in distinguishing the peculiar nature of the lamelliform structures noted in the "neoplastic" cells of the newt's epidermis.

The knot-like structures observed in the tissues treated with hypertonic solutions may be taken first. The difference between these and the lamelliform structures is at once obvious: the membranes of the "knots" are always associated with vacuoles and they look very different in texture from the ergastoplasmic membranes, whereas the lamelliform structures appear identical to the latter except for the absence of the Palade-granules.

Emmelot and Benedetti (1960) have already pointed out the widespread occurrence of concentric orientations of both rough and smooth reticulum, to compare with their own findings. In the adipose cells of the leech *Glossiphonia*, Bradbury and Meek (1958) have shown that the "phospholipid bodies" are made up of purely ergastoplasmic elements. The cytoplasmic lamellar formations observed by Fawcett and Ito (1958) in the cells isolated from the testis of the guinea pig have also the same structures as that of the ordinary ergastoplasm. Differences of the membranous components within a single structure has been pointed out by Haguénau and Lacour (1955) in the "Nebenkern" of a rat's pituitary tumour: here both granule-bearing (or rough) and agranular (or smooth) membranes occur mixed-up.

Of more interest to the present discussion are the recent findings of Emmelot and Benedetti (1960) mentioned above. They observed that the reactions to carcinogen was marked by the formation of smooth-surfaced reticulum which took a dense spongy aspect showing a concentric disposition. The development of smooth reticulum and accumulation of lipid droplets were found to be accompanied by a depletion of glycogen in the cells. In fact, it was in the areas normally occupied by glycogen that the smooth reticulum has been found to develop progressively.

Despite their not showing any preferential disposition to concentric arrangements, the enhanced development of smooth-surfaced reticulum demonstrated by Porter and Bruni (1959) seems relevant to mention in this context. Though the formation of smooth reticulum has been observed in liver cells of fasted animals also, it is shown to be far less in that instance than in the case of the pre-cancerous tissue. The authors consider that the assimilation of carcinogen affects glycogen storage in the liver cells. They further suggest that the enormous

accumulation of lipids in the same cells may "reflect a shift from carbohydrate to lipid metabolism".

Though there exist minor differences between the configurations assumed by smooth reticulum in the two pre-cancerous cases described by Porter-Bruni and Benedetti-Emmelot, and the lamelliform structures of the "neoplasia", the interesting fact emerges that a disturbance of the ergastoplasm resulting in the increased production of smooth reticulum forms a common factor. In all the three cases, direct continuity exists between the smooth reticulum and ergastoplasm. The association of the agranular reticulum with the occurrence of abnormal lipid inclusions is noteworthy. As regards mitochondria, excepting a loss of internal granules noted in the "neoplasia", no other deviation from the normal structure has been observed in these cases.

A curious point which merits a further remark here, is the intimate relation between areas of glycogen and smooth reticulum in the pre-cancerous liver and that of the light areas and lamelliform structures in the "neoplastic" cells of the newt's epidermis. It appears highly suggestive when remembered that up till now in polyester-embedded materials, only polysaccharides have shown a similar lack of electron-density as the light areas. The latter are tentatively considered as possible regions of degeneration. This lends additional support to the view that the lamelliform structures are products of degenerate metabolism. No attempt has been made to ascertain their biochemical nature which, however, will be of extreme interest.

Finally, it needs mention here that in the "12-day-neoplasia," the "new formations" have disappeared much compared to the early stage. On the other hand, vesiculation of the plasma membrane has been seen augmented. Though these tissues have not been so intensely observed as the 6-day cases, the available information shows that degeneration as a result of the action of the carcinogen may have subsided in these tissues.

Now to summarise the views proposed, the enhancement of the inter-cellular spaces, diminution of cellular contacts and the vesiculation of the plasma membrane, all suggest an upset of the normal equilibrium that is supposed to exist between cells in the tissue. Further changes in the cytoplasm described as "new formations" denote a definite disturbance of cellular metabolism, particularly that of lipids and possibly that of proteins and carbohydrates. Such changes have been noted in two pre-cancerous cases and comparable ones in some other instances following abnormal stimulations. The fact that the origin of a temporary neoplasia is marked by similar changes accompanying the genesis of a true cancer is extremely interesting and should incite further work; its resemblance to some other pathological conditions, however, should serve as caution against the pitfalls of premature decisions.

IX. Conclusions

As regards the ordinary cells in the epidermis, the gradual development and transformation (morphogenesis) starting in the basal cells and terminating in the corneal ones are seen associated with definite differences

in cellular contacts: the desmosomes which form specific attachment areas are few and appear labile in the basal region, more numerous and stable upwards, and most developed and greatly extended in the corneal layer. The progressive modifications in desmosomal attachments seem directly referable to the changes in the plasma membrane, though the factors which induce such alterations are not known.

Concerning the clear cells, the classical problem of atypical cells in the amphibian epidermis has been only revived. More detailed observations are necessary to gain proper insight into the roles these cells may play in the economy of the epidermal tissue. Histochemical analyses carried out hand-in-hand with electron microscopy seem highly rewarding.

At the dermo-epidermal junction, the plasma membrane of the basal cells, the adepidermal space and the adepidermal membrane together form a firm complex. The dense thickenings in the plasma membrane here show some similarities to a typical desmosomal moiety. Portions between these thickenings show pinocytosis. It is presumed that the fluid that favours this function is filtered through the adepidermal membrane and space.

As a result of the different kinds of treatments performed, it is concluded that the desmosomes are the least affected structures in the epidermal tissue. This, it may be admitted here, is contrary to the expectations with which the present work was started! An apparent reduction in the number of desmosomes has been noted in the "neoplastic" tissue. As discussed above, if this diminution is confirmed by further quantitative studies, it may support certain interesting deductions: gelation of protoplasm and accompanying contractility; contractility in relation to mutual contact; expenditure of energy in relation to sustenance of contact; and finally, the presumption that desmosomes are gelated, contracted, specific contact areas involved in mutual exchanges of energy.

Most unexpected were the dramatic changes observed in the cytoplasm of the cells from the carcinogen-injected animals. Compared to the results from other experimental treatments in the epidermis, these stand out separate. They show striking similarities to the pre-cancerous changes described by Porter and Bruni (1959) and Emmelot and Benedetti (1960) in the liver cells of rats subjected to the action of different carcinogens. However, as pointed out before, since comparable changes have been shown in connection with other abnormal states, these structural alterations could only be taken as pointers to a degenerate metabolism induced by the carcinogen.

Further work alone can answer the problems raised by the present enquiry. Finer techniques of electron microscopy coupled with histochemical studies can reasonably be expected to yield excellent results. The promise of the epidermis of newt as a fitting tool for probes into the malignant process is well sustained by the present work. It is worth recalling here the remark made by Huxley (1958) while dealing with the biological aspects of cancer: "The advanced state of our analytic knowledge concerning embryology, larval development, metamorphosis and regeneration of Amphibia makes them extremely suitable for cancer research."

X. Summary

A temporary epithelial neoplasia is formed in the newt (*Triturus cristatus* Laur.) when a carcinogen in oil-solution is injected subcutaneously; however, histologically, the component cells of this neoplasia do not betray any structural alteration. To these established facts, the author brings new data showing that the neoplastic cells do manifest modifications observable by electron microscopy.

First, this investigation is concerned with the detailed description of the fine structure of the normal epidermis of the newt. This includes also the ultrastructural characterisation of the different types of cells occurring in the tissue such as the cell of Langerhans, chromatophore cell or the cell of Masson.

Next, the alterations in the fine structure of the epidermis brought about by the action of media of different tonicity are considered. It is noted that the desmosomes remain unaltered in these conditions though many changes are evident in other parts of the tissue.

Finally, the effects of injection of the carcinogenic solution are described. These include alterations of ordinary structures (such as enlargement of intercellular spaces, vesiculation of the plasma membrane, diminution in number of desmosomes) and the appearance of "new formations" (dark bodies considered as lipid droplets, light areas, and lamelliform structures resembling ergastoplasmic vesicles but deprived of ribosomes). The author considers that these "new formations" indicate profound disturbances of cellular metabolism following the assimilation of the carcinogen. These show a remarkable similarity to those structures described by two groups of investigators in the liver cells of rat subjected to the action of different carcinogens.

The present study thus shows a close relationship between submicroscopical changes entailed during the genesis of a benign amphibian neoplasia and those seen during the early stages in the development of a true mammalian cancer.

Résumé

L'injection sous-cutanée d'agents cancérogènes détermine chez le triton (*Triturus cristatus* Laur.) la formation d'une néoplasie épithéliale évidente, mais dont les éléments cellulaires ne semblent pas avoir été morphologiquement affectés. A ces faits admis jusqu'ici, l'auteur apporte des données nouvelles en montrant qu'en réalité les cellules néoplasiques ont subi des modifications de structure que seule la microscopie électronique permet de déceler.

Ce travail complète tout d'abord les connaissances encore insuffisantes que l'on avait sur le plan de l'organisation intime de l'épithélium sain des Batraciens; il précise surtout les caractéristiques de la membrane plasmatique et des desmosomes des différentes cellules de revêtement et définit les particularités submicroscopiques d'éléments ayant fait l'objet de discussion en histologie classique, tels que les cellules de Langerhans et de Masson.

L'auteur examine ensuite quelle est l'action de diverses substances en solutions hypo- ou hypertoniques sur les téguments des Batraciens, et constate que si le tissu

subit différentes altérations, les caractères submicroscopiques des desmosomes ne sont pas modifiés.

L'agent cancérigène provoque une exagération des espaces intercellulaires qui s'accompagne d'une vésiculation des membranes plasmiques et d'une diminution numérique des desmosomes. On constate également, dans le cytoplasme des cellules « néoplasiques », l'apparition de trois types de nouvelles structures, généralement étroitement associées: des corps denses, de nature vraisemblablement lipidique, des zones claires et des structures lamelliformes semblables aux saccules ergastoplasmiques mais dépourvues de ribosomes: l'auteur considère ces néoformations comme l'indice d'une perturbation importante du métabolisme cellulaire. Ces « nouvelles » structures montrent une analogie remarquable avec celles mises en évidence par deux groupes de chercheurs dans les stades initiaux d'hépatomes expérimentaux du rat induits par des cancérigènes chimiques.

Ainsi, l'étude submorphologique de la néoplasie épidermique induite du triton a permis d'établir une relation plus étroite entre ce phénomène bénin et réversible et certains stades initiaux des néoplasies des mammifères.

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